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(54) Modified thermostable DNA polymerase, and DNA polymerase composition for nucleic acid amplification

(57) A modified thermostable DNA polymerase having 5 % or less of the 3'-5' exonuclease activity of the enzyme before modification and a DNA polymerase composition for amplifying nucleic acid, which comprises the modified thermostable DNA polymerase having 0 to 5 % of the 3'-5' exonuclease activity of the enzyme before modification and an unmodified thermostable DNA polymerase having 3'-5' exonuclease activity or a modified thermostable DNA polymerase having 100 to 6 % of the 3'-5' exonuclease activity of a thermostable DNA polymerase before modification; a method for amplifying nucleic acid by use of said modified thermostable polymerase or said DNA polymerase composition; and a reagent therefor.

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Description

The present invention relates to a modified thermostable DNA polymerase, a DNA polymerase composition for amplifying nucleic acid, and a reagent for amplifying nucleic acid containing said enzyme or composition, as well as a method for amplifying nucleic acid by use of said reagent.

Conventionally, a large number of studies have been conducted on thermostable DNA polymerases for use in techniques for amplification of nucleic acid, such as polymerase chain reaction (PCR) etc. Examples of thermostable DNA polymerases used in PCR are DNA polymerase (Tth polymerase) mostly derived from Thermus thermophilus and DNA polymerase (Taq polymerase) derived from Thermus aquaticus. Other known examples are DNA polymerases derived from a hyperthermophilic archaeon strain, such as thermostable DNA polymerase derived from Pyrococcus furiosus (Pfu polymerase, WO92/09689, Unexamined Published Japanese Patent Application No. 328,969/1993) and thermostable DNA polymerase derived from Thermococcus litoralis (Tli polymerase, Unexamined Published Japanese Patent Application No. 7160/1994).

The present inventors have previously found thermostable DNA polymerase excellent in thermostability and DNA extension rate derived from Pyrococcus sp. KOD1 (KOD polymerase, Unexamined Published Japanese Patent Application No. 298,879/1995).

However, these thermostable DNA polymerases have problems such as insufficient amplification of nucleic acid. Further problems with polymerase derived from a hyperthermophilic archaeon strain such as Pyrococcus sp. KOD1 are that it has a 3'-5' exonuclease activity and there is a limit to PCR conditions including reaction time, enzyme amount, primer concentration etc. Therefore, there is demand for novel thermostable DNA polymerase.

As a result of their eager research, the present inventors have succeeded in creating a modified enzyme derived from Pyrococcus sp. KOD1, said enzyme having the 3'-5' exonuclease activity reduced to 5 % or less of the original polymerase before modification while maintaining the DNA extension rate and thermostability of the original polymerase.

The present inventors have further found that the efficiency of amplification using a polymerase before modification is improved by use of its modified thermostable DNA polymerase having a DNA extension rate of at least 30 bases/second and capable of maintaining 60 % or more residual activity at pH 8.8 (determined at 25 °C because it was difficult to measure the pH at 95 °C) after treatment at 95 °C for 6 hours, said modified enzyme having the 3'-5' exonuclease activity reduced to 5 % or less of the polymerase before modification, and the present inventors thereby completed the present invention.

That is, the present invention is a modified thermostable DNA polymerase having the following physicochemical properties:

action:	it has a DNA polymerase activity and has 5 % or less of the 3'-5' exonuclease activity of the enzyme before modification;
DNA extension rate:	at least 30 bases/second; and
thermostability:	it is capable of maintaining 60 % or more residual activity at pH 8.8 (determined at 25 °C) after treatment at 95 °C for 6 hours.

Further, the present invention is a method for amplifying nucleic acid, which comprises reacting DNA as a template, primers, dNTP and the thermostable DNA polymerase of claims 1 to 3, thus extending the primers to synthesize DNA primer extension products.

Further, the present invention is a reagent for amplifying nucleic acid, which comprises 2 kinds of primer, one of which is complementary to a DNA extension product of another primer, dNTP, said thermostable DNA polymerase, and a buffer solution.

As one of methods for amplifying long chain nucleic acid, there is a report on PCR making use of both Taq polymerase (KlenTaq-278) free of 3'-5'exonuclease activity and Pfu polymerase (or Tli polymerase) having 3'-5' exonuclease activity, or of a DNA polymerase composition containing a mixture of their mutant enzymes (Barns, W.M. (1994) Proc. Natl. Acad. Sci. USA 91, 2216-2220).

There is another report on PCR making use of a polymerase composition containing a mixture of Tth polymerase free of 3'-5' exonuclease activity, Pfu polymerase (or Tli polymerase) with 3'-5' exonuclease activity, and thermostable DNA polymerase derived from Thermotoga maritima (Unexamined Published Japanese Patent Application No. 38198/1996).

Higher efficiency of amplification can be attained by such a composition than by one kind of DNA polymerase but is still not sufficient because 2 kinds of DNA polymerase having different properties in thermostability and DNA extension rate are used. Hence, there has been demand for a method further excellent in effici ncy of amplification.

As a result of their eager research under these circumstances, the present inventors found that PCR excellent in efficiency of amplification can be effected using a DNA polymerase composition for nucleic acid amplification, consist-

ing of a combination of first and second DNA polymerases being almost identical to each other with respect to thermostability and DNA extension rate, the activity of the second DNA polymerase being present at a lower level than that of the first DNA polymerase, specifically a DNA polymerase composition comprising DNA polymerases selected from the group consisting of a modified thermostable DNA polymerase (first polymerase) having 0 to 5 % of the 3'-5' exonuclease activity of the naturally occurring enzyme before modification and a modified thermostable DNA polymerase (second polymerase) having 100 to 6 % of the 3'-5' exonuclease activity of a naturally occurring DNA polymerase or of its original naturally occurring enzyme before modification.

That is, the present invention is a DNA polymerase composition for amplifying nucleic acid, which comprises a modified thermostable DNA polymerase having 0 to 5 % of the 3'-5' exonuclease activity of the original enzyme before modification (first polymerase) and the original enzyme or a modified thermostable DNA polymerase having 100 to 6 % of the 3'-5' exonuclease activity of its original thermostable enzyme before modification (second polymerase), said first and second DNA polymerases having a DNA extension rate of at least 30 bases/second and capable of maintaining 60 % or more residual activity at pH 8.8 (determined at 25 °C) after treatment at 95 °C for 6 hours.

Further, the present invention is a method for amplifying nucleic acid, which comprises reacting DNA as a template, primers, dNTP and said DNA polymerase composition, thus extending the primers to synthesize a DNA primer extension product.

Further, the present invention is a reagent for amplifying nucleic acid, which comprises 2 kinds of primer, one of which is complementary to a DNA extension product of another primer, dNTP, said DNA polymerase composition, divalent ions, monovalent ions, and a buffer solution.

FIG. 1 shows the polymerase activity of the modified DNA polymerase and degree of decomposition of DNA.

FIG. 2 shows the thermostability of the modified DNA polymerase.

FIG. 3 shows the result of PCR by use of the modified DNA polymerase (for plasmid).

FIG. 4 shows the result of PCR by use of the modified DNA polymerase (for human genome).

FIG. 5 shows the result of PCR by use of the DNA polymerase composition (for human genome).

FIG. 6 shows the amino acid sequences of the exo regions of the thermostable DNA polymerase.

FIG. 7 shows the polymerase activity of the modified DNA polymerase and degree of decomposition of DNA.

FIG. 8 shows polymerase activity relative to the naturally occurring KOD polymerase.

In the present invention, DNA polymerase activity refers to a catalytic activity to introduce deoxyribonucleoside-5'-monophosphate template-dependently into deoxyribonucleic acid by covalently binding the α-phosphate of deoxyribonucleoside-5'-triphosphate to the 3'-hydroxyl group of an oligonucleotide or polynucleotide annealed to a template DNA.

If the enzyme activity in a sample is high, activity measurement shall be carried out after the sample is diluted with a preserving buffer solution. In the present invention, 25 μl of Solution A below, 5 μl each of Solutions B and C below, and 10 μl of sterilized water are added to an Eppendorf tube, then stirred and mixed, and 5 μl of the above enzyme solution is added to it and reacted at 75 °C for 10 minutes. Thereafter, the sample is cooled on ice, and 50 μl of Solution E and 100 μl of Solution D below are added to it, then stirred, and cooled on ice for 10 minutes. The solution is filtered through a glass filter (Wattman GF/C Filter), and the filter is washed intensively with Solution D and ethanol, and the radioactivity of the filter is counted in a liquid scintillation counter (Packard) to determine the incorporation of the nucleotide into the template DNA. In the present invention, 1 unit of the enzyme activity shall be defined as the amount of the enzyme causing 10 nmol nucleotide per 30 minutes to be incorporated into the acid insoluble fragment under these conditions.

A: 40 mM Tris-HCl (pH 7.5)
16 mM magnesium chloride
15 mM dithiothreitol
100 μg/ml BSA

B: 2 μg/μl activated calf thymus DNA

C: 1.5 mM dNTP (250 cpm/pmol [^{3}H] dTTP)

D: 20 % trichloroacetic acid (2 mM sodium pyrrophosphate)

E: 1 μg/μl carrier DNA

In the present invention, the 3'-5' exonuclease activity refers to the activity of deleting a 3'-terminal region of DNA to deliver 5'-mononucleotide to a template.

The activity measurement method is as follows: 50 μl reaction solution (120 mM Tris-HCl (pH 8.8 at 25 °C), 10 mM KCl, 6 mM ammonium sulfate, 1 mM $MgCl_2$, 0.1 % Triton X-100, 0.001 % BSA, 5 μg of E. coli DNA labeled with tritium) is pipetted into a 1.5 ml Eppendorf tube, followed by adding DNA polymerase to it. After the mixture is reacted at 75 °C for 10 minutes, the reaction is terminated by cooling on ice. Then, 50 μl of 0.1 % BSA is added to it as a carrier, and then 100 μl of a solution containing 10 % trichloroacetic acid and 2 % sodium pyrrophosphate is mixed with it. After the mixture is left on ice for 15 minutes, it is centrifuged at 12,000 r.p.m. for 10 minutes to separate precipitates. 100 μl of

the supernatant is measured for radioactivity in a liquid scintillation counter (Packard) whereby the amount of the nucleotide delivered to the acid soluble fragment is determined.

In the present invention, DNA extension rate refers to the number of DNAs extended per unit time. The measurement method is as follows: A reaction solution of DNA polymerase (20 mM Tris-HCl (pH 7.5), 8 mM magnesium chloride, 7.5 mM dithiothreitol, 100 μg/ml BSA, 0.1 mM dNTP, 0.2 μCi [α-^{32}P]dCTP) is reacted at 75 °C with a single-stranded chain of M13mp18 DNA to which a prier had been annealed. The reaction is terminated by adding an equal volume of a reaction terminating solution (50 mM sodium hydroxide, 10 mM EDTA, 5 % Ficoll, 0.05 Bromophenol Blue). The DNA extended by the reaction is fractionated by electrophoresis on alkali agarose gel, and the gel is dried and subjected to autoradiography. As the DNA size marker, labeled λ/HindIII is used. The DNA extension rate is determined on the basis of the DNA size as determined with a band of this marker as an indicator.

In the present invention, thermostability means residual activity at pH 8.8 (the pH value determined at 25 °C) after treatment at 95 °C for 6 hours.

One embodiment of the present invention is a modified thermostable DNA polymerase having the following physicochemical properties:

action:	it has a DNA polymerase activity and has 5 % or less of the 3'-5' exonuclease activity of the original enzyme before modification,
DNA extension rate:	at least 30 bases/second,
thermostability:	it is capable of maintaining residual activity at pH 8.8 (determined at 25 °C) after treatment at 95 °C for 6 hours,
optimum temperature:	about 75 °C,
molecular weight:	88 to 90 kDa, and
amino acid sequence:	an amino acid sequence as shown in SEQ ID NO: 2 in which at least one of amino acids at the 141-, 143-, 210- and 311-positions has been replaced by another amino acid.

Another embodiment of the present invention is a modified thermostable DNA polymerase having the following physicochemical properties:

action:	it has a DNA polymerase activity and is free of a 3'-5' exonuclease activity,
DNA extension rate:	at least 30 bases/second,
thermostability:	it is capable of maintaining 60 % or more residual activity at pH 8.8 (determined at 25 °C) after treatment at 95 °C for 6 hours,
optimum temperature:	about 75 °C,
molecular weight:	88 to 90 kDa, and
amino acid sequence:	an amino acid sequence as shown in SEQ ID NO: 2 in which at least one of amino acids at the 141-, 143-, 210- and 311-positions has been replaced by another amino acid.

The thermostable DNA polymerase of the present invention before modification is an enzyme derived from Pyrococcus sp. KOD as a hyperthermophilic archaeon strain isolated in Kodakara Island, Kagoshima prefecture, Japan. The microbial properties of KOD producing this enzyme is described in Unexamined Published Japanese Patent Application No 298,879/1995. This enzyme is produced by culturing this strain.

This enzyme has the following physicochemical properties:

action:	it has a DNA polymerase activity and has a 3'-5' exonuclease activity,
DNA extension rate:	at least 120 bases/second,
thermostability:	it is capable of maintaining 60 % or more residual activity at pH 8.8 (determined at 25 °C) after treatment at 95 °C for 6 hours,
optimum temperature:	about 75 °C,
molecular weight:	88 to 90 kDa, and
amino acid sequence:	the amino acid sequence of SEQ ID NO: 2.

The preferable thermostable DNA polymerase of the present invention has an amino acid sequence as shown in SEQ ID NO: 2 in which at least one of amino acids at the 141-, 143-, 210- and 311-positions has been replaced by another amino acid. One example is the enzyme where in SEQ ID NO: 2 aspartic acid at the 141-position has been replaced by alanine; glutamic acid at the 143-position by alanine; aspartic acid at the 141-position and glutamic acid at

the 143-position respectively by alanine; asparagine at the 210-position by aspartic acid; or tyrosine at the 311-position by phenylalanine.

For production of these modified enzymes, there is a method in which a gene coding for naturally occurring KOD polymerase is mutated so that a novel enzyme having a lower 3'-5' exonuclease activity than the naturally occurring KOD polymerase is produced by protein engineering.

Although the KOD polymerase-coding gene to be mutated is not particularly limited, a gene defined in SEQ ID NO: 3 in the Sequence Listing, derived from Pyrococcus sp. KOD, was used in one embodiment of the present invention.

A DNA polymerase gene derived from the KOD1 strain contains 2 intervening sequences (1374 to 2453 bp and 2708 to 4316 bp), and therefore a modified DNA polymerase having 3'-5' exonuclease activity reduced can be obtained by e.g. preparing with a PCR fusion method a mature gene having a nucleotide sequence as shown in SEQ ID NO: 3 from a gene as shown in SEQ ID NO: 1 and using the thus prepared gene.

In another embodiment of the present invention, a novel enzyme with a less 3'-5' activity than the naturally occurring KOD polymerase is produced by mutating a gene coding for the amino acid sequence of SEQ ID NO: 1.

To mutate the naturally occurring KOD polymerase gene, any of the known methods can be used. For example, use can be made of methods which involve bringing a drug as a mutagen into contact with the naturally occurring KOD polymerase gene or irradiating the gene with UV ray, or of protein engineering means such as the PCR technique or site specific mutagenesis. E. coli, whose gene undergoes mutations frequently because its mismatch repair is destroyed, can also be used for in vivo mutation.

The chameleon site-directed mutagenesis kit (Stratagene) used in the present invention make use of the following steps: (1) denaturing a plasmid having a target gene inserted into it and then annealing a mutagenesis primer and a selective marker to said plasmid, (2) extending DNA by a DNA polymerase and then conducting ligation reaction using a ligase, (3) cleaving the plasmid with a restriction enzyme whose restriction site is not present in the selective primer but present in the plasmid serving as a template, whereby DNA which was not mutated is cleaved, (4) transforming E. coli with the remaining plasmid, (5) preparing the mutant plasmid from the transformant, followed by conducting (3) and (4) repeatedly so that the plasmid mutated as desired is obtained.

The modified polymerase gene obtained as described above is transformed into e.g. E. coli and then plated on a agar medium containing a drug such as ampicillin to form a colony. The colony is inoculated onto a nutrient medium such as LB medium or 2 x YT medium, then cultured at 37 °C for 12 to 20 hours, and disrupted so that a crude enzyme solution is extracted from it.

To disrupt the microorganism, any of the known means by physical disruption by ultrasonication or glass beads or with lytic enzyme such as lysozyme can be used. The crude enzyme is thermally treated e.g. at 80 °C for 30 minutes to inactivate the polymerases originating in the host. Then, its DNA polymerase activity is determined and its 3'-5' exonuclease activity is determined and their activity ratio is determined. Then, this ratio is compared with that of the naturally occurring KOD polymerase in order to screen the enzyme having a reduced 3'-5' exonuclease activity.

From the strain selected in this manner, the DNA polymerase can be purified using any of the known means, for example as follows:

The microorganism cultured in a nutrient medium is recovered and disrupted enzymatically or by physical means so that a crude enzyme is extracted. The crude enzyme extract is subjected to heat treatment e.g. at 80 °C for 30 minutes and thereafter the KOD polymerase fraction is recovered by precipitation with sulfate ammonium. This crude enzyme fraction can be desalted by e.g. gel filtration on Sephadex G-25 (Pharmacia Biotech).

After this procedure, a purified enzyme preparation can be obtained by chromatography such as Q-Sepharose, heparin-Sepharose etc. In this process, the enzyme preparation can be purified to such a degree that it shows an almost single band in SDS-PAGE.

A DNA primer extension product can be produced using the modified thermostable DNA polymerase of the present invention by reacting primers and dNTP with DNA as a template to extend the primers. The primers are 2 kinds of oligonucleotide, one of which is preferably a primer complementary to a DNA extension product of another primer. Heating and cooling are carried out repeatedly.

Magnesium ions and ammonium ions and/or potassium ions are preferably coexistent for the DNA polymerase of the present invention to maintain its activity. The PCR reaction solution may further contain a buffer solution and these ions along with BSA and a nonionic surface active agent such as Triton X-100 in the buffer solution.

Because the 3'-5' exonuclease activity of the modified thermostable DNA polymerase of the present invention is reduced as compared with the enzyme before modification, PCR can be effected with higher efficiency of amplification by the modified thermostable DNA polymerase than by the enzyme before modification.

Hereinafter, the composition of at least 2 kinds of thermostable DNA polymerase which are different in their 3'-5' exonuclease activity is described.

A first DNA polymerase of the present invention is an enzyme having a 3'-5' exonuclease activity reduced to 0 to 5 % preferably 1 % or less of the 3'-5' exonuclease activity of the enzyme before modification.

The first DNA polymerase includes an enzyme having an amino acid sequence as shown in SEQ ID NO: 2 in which

at least one of amino acids at the 141-, 142-, 143-, 210- and 311-positions has been replaced by another amino acid. One example is an enzyme having an amino acid sequence as shown in SEQ ID NO: 2 in which aspartic acid at the 141-position has been replaced by alanine; glutamic acid at the 143-position by alanine; aspartic acid at the 141-position and glutamic acid at the 143-position respectively by alanine; asparagine at the 210-position by aspartic acid; or tyrosine at the 311-position by phenylalanine. Further, it includes the enzyme where isoleucine at the 142-position has been replaced by arginine.

The first DNA polymerase of the present invention includes a modified thermostable DNA polymerase having the following physicochemical properties:

action:	it has a DNA polymerase activity and has 0 to 5 % of the 3'-5' exonuclease activity of the enzyme before modification;
DNA extension rate:	at least 30 bases/second; and
thermostability:	it is capable of maintaining 60 % or more residual activity at pH 8.8 (determined at 25 °C) after treatment at 95 °C for 6 hours.

The first DNA polymerase of the present invention further includes a modified thermostable DNA polymerase having the following physicochemical properties:

action:	it a DNA polymerase activity and has 0 to 5 % of the 3'-5' exonuclease activity of the enzyme before modification;
DNA extension rate:	at least 30 bases/second;
thermostability:	it is capable of maintaining residual activity at pH 8.8 (determined at 25 °C) after treatment at 95 °C for 6 hours;
optimum temperature:	about 75 °C;
molecular weight:	88 to 90 kDa; and
amino acid sequence:	an amino acid sequence as shown in SEQ ID NO: 2 in which at least one of amino acids at the 141-, 142-, 143-, 210- and 311-positions has been replaced by another amino acid.

The first DNA polymerase of the present invention further includes a modified thermostable DNA polymerase having the following physicochemical properties:

action:	it has a DNA polymerase activity and has 0 to 5 % of the 3'-5' exonuclease activity of the enzyme before modification;
DNA extension rate:	at least 30 bases/second;
thermostability:	it is capable of maintaining 60 % or more residual activity at pH 8.8 (determined at 25 °C) after treatment at 95 °C for 6 hours;
optimum temperature:	about 75 °C;
molecular weight:	88 to 90 kDa; and
amino acid sequence:	an amino acid sequence as shown in SEQ ID NO: 2 in which aspartic acid at the 141-position has been replaced by alanine; isoleucine at the 142-position by arginine; glutamic acid at the 143-position by alanine; aspartic acid at the 141-position and glutamic acid at the 143-position respectively by alanine; asparagine at the 210-position by aspartic acid; or tyrosine at the 311-position by phenylalanine.

The second DNA polymerase of the present invention includes a modified thermostable polymerase having 100 to 6 % preferably 90 to 30 % of the 3'-5' exonuclease activity of a thermostable DNA polymerase having a 3'-5' exonuclease activity or the original unmodified thermostable DNA polymerase having a 3'-5' exonuclease activity. The second DNA polymerase includes e.g. the enzyme with the amino acid sequence of SEQ ID NO: 2 or with an amino acid sequence as shown in SEQ ID NO: 2 in which amino acids at the 140-, 142-, or 144-position have been replaced by other amino acids. One example is the enzyme with an amino acid sequence as shown in SEQ ID NO: 2 in which isoleucine at the 142-position has been replaced by aspartic acid, glutamic acid, asparagine, glutamine or lysin, or threonine at the 144-position by valine.

The second DNA polymerase of the present invention includes a modified thermostable DNA polymerase having the following physicochemical properties:

action:	it has has a DNA polymerase activity and has a 3'-5' exonuclease activity;

DNA extension rate:	at least 30 bases/second;
thermostability:	it is capable of maintaining 60 % or more residual activity at pH 8.8 (determined at 25 °C) after treatment at 95 °C for 6 hours;
optimum temperature:	about 75 °C;
molecular weight:	88 to 90 kDa; and
amino acid sequence:	
	the amino acid sequence of SEQ ID NO: 2.

The second DNA polymerase of the present invention further includes a modified thermostable polymerase having the following physicochemical properties:

action:	it has a DNA polymerase activity and has 100 to 6 % preferably 90 to 30 % of the 3'-5' exonuclease activity of the enzyme before modification;
DNA extension rate:	at least 30 bases/second; and
thermostability:	it is capable of maintaining 60 % or more residual activity at pH 8.8 (determined at 25 °C) after treatment at 95 °C for 6 hours; and
amino acid sequence:	
	an amino acid sequence as shown in SEQ ID NO: 2 in which at least one of amino acids X_1, X_2 and X_3 in an $X_1DX_2EX_3$ motif present in EXO 1 has been replaced by another amino acid.

In the amino acid sequence of the DNA polymerase with a 3'-5' exonuclease activity, highly preserving amino acid regions for this exonuclease activity are known (EXO I, EXO II and EXO III, FIG 6). EXO I region contains an $X_1DX_2EX_3$ motif, and the amino acids D (aspartic acid) and E (glutamic acid) are known to be essential for the exonuclease activity.

The second DNA polymerase of the present invention further includes a modified thermostable DNA polymerase having the following physicochemical properties:

action:	it has a DNA polymerase activity and has 100 to 6 % preferably 90 to 30 % of the 3'-5' exonuclease activity of the enzyme before modification;
DNA extension rate:	at least 30 bases/second;
thermostability:	it is capable of maintaining 60 % or more residual activity at pH 8.8 (determined at 25 °C) after treatment at 95 °C for 6 hours; and
amino acid sequence:	
	an amino acid sequence as shown in SEQ ID NO: 2 in which amino acids at 140-, 142-, or 144-position have been replaced by other amino acids.

The second DNA polymerase of the present invention further includes a modified thermostable DNA polymerase having the following physicochemical properties:

action:	it has a DNA polymerase activity and has 100 to 6 % preferably 90 to 30 % of the 3'-5' exonuclease activity of the enzyme before modification;
DNA extension rate:	at least 30 bases/second;
thermostability:	it is capable of maintaining 60 % or more residual activity at pH 8.8 (determined at 25 °C) after treatment at 95 °C for 6 hours;
optimum temperature:	about 75 °C;
molecular weight:	88 to 90 KDa; and
amino acid sequence:	
	an amino acid sequence as shown in SEQ ID NO: 2 in which is oleucine at the 142-position has been replaced by aspartic acid, glutamic acid, asparagine, glutamine or lysin, or threonine at the 144-position by valine.

The DNA extension rate of the first and second DNA polymerases is at least at least 30 bases/second, preferably 100 to 120 bases/second and they are thermostable DNA polymerases capable of maintaining 60 % or more residual activity at pH 8.8 (determined at 25 °C) after treatment at 95 °C for 6 hours.

The first and second DNA polymerases are preferably KOD polymerases or their mutants.

In the present invention, the activity of the second DNA polymerase is preferably low than that of the first DNA polymerase, and the second DNA polymerase is preferably 0.02 to 0.1 unit every 2.5 units of the first DNA polymerase.

To produce these modified enzymes, there is a method in which a gene coding for e.g. naturally occurring KOD polymerase is mutated so that the novel enzymes having reduced 3'-5' exonuclease activity as compared with the nat-

urally occurring KOD polymerase are produced by protein engineering means.

The KOD polymerase-coding gene to be mutated is not particularly limited. In one embodiment of the present invention, the gene shown in SEQ ID NO: 3 in the Sequence Listing, derived from Pyrococcus sp. KOD, was used.

In another embodiment of the present invention, a gene coding for the amino acid sequence of SEQ ID NO: 1 is mutated to produce the novel enzyme having the 3'-5' exonuclease activity reduced as compared with that of the naturally occurring KOD polymerase.

The thermostable DNA polymerase of the present invention before modification is an enzyme derived from Pyrococcus sp. KOD as 1 kind of hyperthermophilic archaeon strain isolated in Kodakara Island, Kagoshima prefecture, Japan. The microbial properties of KOD producing said enzyme are described in Unexamined Published Japanese Patent Application No 298,879/1995. Said enzyme is produced by culturing this strain.

This enzyme has the following physicochemical properties:

action:	it has a DNA polymerase activity and has a 3'-5' exonuclease activity;
DNA extension rate:	at least 120 bases/second;
thermostability:	it is capable of maintaining 60 % or more residual activity at pH 8.8 (determined at 25 °C) after treatment at 95 °C for 6 hours;
optimum temperature:	about 75 °C;
molecular weight:	88 to 90 kDa; and
amino acid sequence:	the amino acid sequence of SEQ ID NO: 2.

The method of amplifying nucleic acid according to the present invention comprises reacting DNA as a template, primers, and 4 kinds of deoxyribonucleotide triphosphate (dNTP) by use of said DNA polymerase composition, thus extending the primers to synthesize a DNA primer extension product.

In the PCR techniques as one method of amplifying nucleic acid according to the present invention, if a target nucleic acid in a sample is particularly long and double-stranded, then it is denatured by heating to be separated into single-stranded chains. If separation of the long chain nucleic acid into single-stranded chains is inadequate, subsequent annealing and extension reaction of the primers will be prevented. Then, the single-stranded chains as a template, primers complementary to said template, preferably primers one of which is complementary to another DNA extension product, and dNTP are reacted in a PCR reaction solution using the DNA polymerase composition of the present invention.

This reaction is carried out using a 2-stage temperature cycle, that is a high temperature stage for denaturing the nucleic acid to be amplified and a low temperature stage for annealing the primers to the denatured nucleic acid to initiate primer extension, and this cycle is repeated 25 to 40 times. Usually, 1 cycle consists of reaction at 94 °C for 0.5 to 1 minute and then at 68 °C for 0.5 to 10 minutes. The 2 primers are annealed to opposite strands of the template nucleic acid sequence, and an extension product starting at each primer is a copy complementary to the template nucleic acid, and the product is oriented so that it can hybridize to another primer when separated from the resulting double-stranded chain.

The reaction time is conducted preferably for a sufficient period until the extension reaction completes chain extension. For amplification of 20 kb or more nucleic acid, an annealing and extension time of at least 10 to 20 minutes is preferable.

A long chain nucleic acid is preferably protected from decomposition during amplification by using e.g. glycerol, dimethyl sulfoxide (DMSO) etc.

The presence of a misincorporated nucleotide will finish chain extension earlier and the number of template chains for subsequent amplification will be decreased, resulting in reduction of efficiency of amplification of long chain nucleic acid. In the present invention, however, the nucleotide misincorporated during synthesis of a primer extension product will be removed because the 3'-5' exonuclease activity besides the DNA polymerase activity is present at a low level in the reaction solution, and the dominant polymerase activity enables complete chain extension.

The pH and composition for a reaction buffer, salts (divalent and monovalent ions), and the design of primers are important for efficiency of amplification of long chain nucleic acid.

Because the PCR reagent is prepared usually at room temperature before the denaturation step, the binding of primers to another primer or to a homologous part of a nucleic acid sequence may be caused. If an extension product is also formed by nonspecific binding of primers, the efficiency of amplification of the desired long chain product is reduced. To prevent nonspecific binding, "hot start method" such as addition of the enzyme after the reaction solution reaches a high temperature is preferably used.

Divalent ions e.g. magnesium ions and monovalent ions e.g. ammonium and/or potassium ions are preferably allowed to coexist to maintain the activity of the DNA polymerase of the present invention. Further, a buffer solution, such ions, BSA, a nonionic surface active agent (e.g. Triton X-100) and buffer solution may be present in the reaction

solution for nucleic acid amplification.

The reagent for amplifying nucleic acid according to the present invention contains 2 primers, one of which is complementary to a DNA extension product of another primer, dNTP, said DNA polymerase composition, magnesium ions, ammonium ions and/or potassium ions, BSA, a nonionic surface active agent and a buffer solution.

In the present invention, the activity of the second DNA polymerase is preferably lower than that of the first DNA polymerase, and it is preferable that the second DNA polymerase is present in 0.02 to 0.1 unit every 2.5 units of the first DNA polymerase.

The reagent of the present invention may contain a solvent aid such as glycerin, DMSO, polyethylene glycol etc.

The buffer solution used includes a tris buffer, tris(hydroxymethyl)methylglycine (tricine buffer), N-bis(hydroxyethyl)glycine (bicine buffer) etc. The optimum buffer solution and pH depend on the DNA polymerase used. If KOD polymerase or its mutant is used in the present invention, a buffer solution is used at pH 7.5 to 9.2 (at 25 °C) at concentration of 10 to 50 mM, preferably 20 to 120 mM. Divalent cations are preferably magnesium ions, and magnesium chloride etc. are used. Their concentration is preferably 1 to 2 mM. Monovalent cations are preferably ammonium ions or potassium ions, and ammonium sulfate, potassium glutamate, potassium acetate etc. are used. Their concentration is preferably 2 to 50 mM. The primers used are 2 kinds of oligonucleotide, one of which is a primer complementary to a DNA extension product of another primer. Their concentration is preferably 0.2 to 1 μM.

Hereinafter, the present invention is described in detail with reference to the Examples.

Reference Example 1.

Cloning of DNA Polymerase Gene Derived from Hyperthermophilic Archaeon Strain KOD

Hyperthermophilic archaeon strain KOD1 isolated in Kodakara Island, Kagoshima Prefecture, Japan, was cultured at 95 °C and then recovered. Genomic DNA of hyperthermophilic archaeon strain KOD was prepared in a usual manner from the microorganism. Two primers, were synthesized on the basis of preserving regions in the amino acid sequence of DNA polymerase (Pfu polymerase) derived from Pyrococcus furiosus. PCR was conducted using the 2 primers and the genomic DNA as a template.

The DNA fragment thus amplified by PCR was sequenced, and from the nucleotide sequence thus determined, its amino acid sequence was deduced. Then, the genomic DNA from the KOD1 strain was treated with restriction enzyme, and the di gest was subjected to Southern hybridization with the above amplification DNA fragment as a probe to determine the size of a fragment coding for the DNA polymerase (about 4 to 7 kbp). Further, the DNA fragment of this size was recovered from the corresponding agarose gel and inserted into plasmid pBS (Stratagene). The mixture thus obtained was transformed into E. coli JM109 to prepare a library. Colony hybridization with the same probe as in the Southern hybridization was conducted so that a clone strain (E. coli JM109/pSBKOD1) considered to contain the DNA polymerase gene derived from the KOD1 strain was obtained from the library.

Plasmid pSBKOD1 was recovered from the clone strain (E. coli JM109/pSBKOD1) and sequenced in a usual manner. Its amino acid sequence was deduced from the nucleotide sequence thus determined. The DNA polymerase gene derived from the KOD1 strain consisted of 5010 bases and coded for 1670 amino acids (SEQ ID NO: 1).

To prepare a complete polymerase gene, 2 intervening sequences (1374 to 2453 bp and 2708 to 4316 bp) were removed by a PCR fusion method. Three pairs of primers were used in the PCR fusion method and each pair was used in PCR where the plasmid recovered from the cloned strain was used as a template, so that 3 fragments free of the intervening sequences were amplified. The primers used in PCR were designed such that they have the same sequence as a sequence binding to the target site, and that they have different restriction enzyme sites at the terminals, that is, they have an EcoRV site at the N-terminal and a BamHI site at the C-terminal. Then, a fragment located in the middle of the PCR amplification fragment was mixed with a fragment located at the N-terminal side, and PCR was conducted using the respective fragments as primers. Further, a fragment located in the middle of the PCR amplification fragment was mixed with a fragment located at the C-terminal side, and PCR was conducted using the respective fragments as primers. PCR was conducted again using the 2 kinds of fragment thus obtained to give a complete gene fragment which is free of the intervening sequences, has an EcoRV site at the N-terminal and a BamHI site at the C-terminal, and codes for the DNA polymerase derived from the KOD1 strain. Further, this gene was subcloned in expression vector PET-8c capable of inducing expression of the gene under T7 promoter. For this subcloning, the NcoI/BamHI sites on PET-8c and the restriction enzyme sites created above were used. A recombinant expression vector (pET-pol) was thus obtained. E. coli BL21 (DE3)/pET-pol has been deposited as FERM BP-5513 with the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan.

Example 1.

Subcloning of the KOD Polymerase Gene

To modify thermostable DNA polymerase, the KOD polymerase gene was removed from plasmid pET-pol and subcloned in plasmid pBluescript as follows:

The KOD polymerase gene, about 2.3 kb long, was removed by digesting plasmid pET-pol with restriction enzymes XbaI and BamHI (Toyobo Co., Ltd.). A ligation kit (Ligation high, a product of Toyobo Co., Ltd.) was then used for ligation of this DNA fragment into plasmid pBluescript SK(-) previously digested with XbaI and BamHI. Then, the resulting plasmid was transformed into commercially available competent cells (competent high JM109, available from Toyobo Co., Ltd.).

The transformant was cultured at 35 °C for 16 hours in an LB agar medium containing 100 μg/ml ampicillin (1 % Bacto-trypton, 0.5 % yeast extract, 0.5 % sodium chloride, 1.5 % agar, produced by Gibco), and a plasmid was prepared from the resulting colonies. From its partial nucleotide sequence, this plasmid was confirmed to carry the KOD polymerase gene and it was designated plasmid pKOD1.

Example 2.

Preparation of Modified Gene (DA) and Purification of Modified Thermostable DNA Polymerase

Plasmid pKOD1 obtained in Example 1 was used to prepare a plasmid (pKODDA) carrying a gene for a modified thermostable DNA polymerase of the KOD polymerase of SEQ ID NO: 2 in which aspartic acid at the 141-position had been replaced by alanine.

For this preparation, a chameleon site-directed mutagenesis kit (Stratagene) was used according to the manufacture's instructions.

The selective primer used was a primer as shown in SEQ ID NO: 4. The mutagenesis primer used was a primer as shown in SEQ ID NO: 7. The mutant was confirmed by determining its nucleotide sequence. E. coli JM109 was transformed with the resulting plasmid to give JM109 (pKODDA).

6 L sterilized TB medium (described in p. A. 2 in Molecular cloning) containing 100 μg/ml ampicillin was introduced into a 10-L jar fermenter. Into this medium was inoculated E. coli JM109 (pKODDA) which had been cultured at 30 °C for 16 hours in 50 ml LB medium (1 % Bacto-trypton, 0.5 % yeast extract, 0.5 % sodium chloride, produced by Gibco) containing 100 μg/ml ampicillin in a 500-ml flask, and the microorganism was grown by shake culture at 35 °C for 12 hours under aeration. The microorganism was recovered from the culture by centrifugation, then suspended in 400 ml buffer (10 mM Tris-HCl (pH 8.0), 80 mM KCl, 5 mM 2-mercaptoethanol, 1 mM EDTA) and disrupted by ultrasonication to give a cell lysate.

The cell lysate was heated at 85 °C for 30 minutes and centrifuged to remove insoluble solids. The supernatant was treated with polyethylene imine for removal of nucleic acids, then fractionated with sulfate ammonium and subjected to chromatography on heparin-Sepharose. Finally, the buffer solution was replaced by a preserving buffer solution (50 mM Tris-HCl (pH 8.0), 50 mM potassium chloride, 1 mM dithiothreitol, 0.1 % Tween 20, 0.1 % Nonidet™ P40, 50 % glycerin) so that the modified thermostable DNA polymerase (DA) was obtained.

In the purification described above, the measurement of DNA polymerase activity was conducted in the following manner. When the enzyme activity was high, the sample was measured after dilution with the preserving buffer solution.

(Reagent)

A: 40 mM Tris-HCl (pH 7.5)
16 mM magnesium chloride
15 mM dithiothreitol
100 μg/ml BSA
B: 2 μg/μl activated calf thymus DNA
C: 1.5 mM dNTP (250 cpm/pmol [^{3}H] dTTP)
D: 20 % trichloroacetic acid (2 mM sodium pyrrophosphate)
E: 1 μg/μl carrier DNA

(Method)

25 μl of Solution A, 5 μl each of Solutions B and C, and 10 μl sterilized water are added to an Eppendorf tube and

mixed by stirring. Then, 5 µl of the enzyme solution is added to the mixture and reacted at 75 °C for 10 minutes. Thereafter, it is cooled on ice for 10 minutes, followed by adding 50 µl of Solution E and 100 µl of Solution D. The mixture was stirred and cooled on ice for 10 minutes. This solution is filtered through a glass filter (Wattman GF/C filter), followed by extensive washing with Solution D and ethanol, and the radioactivity of the filter was counted in a liquid scintillation counter (Packard) to determine the incorporation of the nucleotide into the template DNA.

1 unit of the enzyme is assumed to be the amount of the enzyme causing incorporation, into the acid insoluble fragment, of 10 nmol nucleotide every 30 minutes under these conditions.

Example 3.

Preparation of Modified Gene (EA) and Purification of Modified Thermostable DNA Polymerase

A plasmid (pKODEA) carrying a gene for a modified thermostable DNA polymerase of the KOD polymerase of SEQ ID NO: 2 in which glutamine at the 143-position had been replaced by alanine was prepared in the same manner as in Example 2.

The selective primer used was a primer as shown in SEQ ID NO: 5. The mutagenesis primer used was a primer as shown in SEQ ID NO: 8. The modified thermostable DNA polymerase (EA) was obtained using the same purification method as in Example 2.

Example 4.

Preparation of Modified Gene (DEA) and Purification of Modified Thermostable DNA Polymerase

A plasmid (pKODDEA) carrying a gene for a modified thermostable DNA polymerase of the KOD polymerase of SEQ ID NO: 2 in which aspartic acid at 141-position and glutamic acid at the 143-position had been replaced by alanine respectively was prepared in the same manner as in Example 2. The selective primer used was a primer as shown in SEQ ID NO: 4. The mutagenesis primer used was a primer as shown in SEQ ID NO: 6. The modified thermostable DNA polymerase (DEA) was obtained using the same purification method as in Example 2.

Example 5.

Preparation of Modified Gene (ND) and Purification of Modified Thermostable DNA Polymerase

A plasmid (pKODND) carrying a gene for a modified thermostable DNA polymerase of the KOD polymerase of SEQ ID NO: 2 in which asparagine at 210-position had been replaced by aspartic acid was prepared in the same manner as in Example 2. The selective primer used was a primer as shown, in SEQ ID NO: 4. The mutagenesis primer used was a primer as shown in SEQ ID NO: 9. The modified thermostable DNA polymerase (ND) was obtained using the same purification method as in Example 2.

Example 6.

Preparation of Modified Gene (YF) and Purification of Modified Thermostable DNA Polymerase

A plasmid (pKODYF) carrying a gene for a modified thermostable DNA polymerase of the KOD polymerase of SEQ ID NO: 2 in which tyrosine at 311-position had been replaced by phenylalanine was prepared in the same manner as in Example 2. The selective primer used was a primer as shown in SEQ ID NO: 4. The mutagenesis primer used was a primer as shown in SEQ ID NO: 10. he modified thermostable DNA polymerase (YF) was obtained using the same purification method as in Example 2.

Example 7.

Confirmation of Exonuclease Activity of Modified Thermostable DNA Polymerase

The exonuclease activities of the modified thermostable DNA polymerases (DA, EA, DEA, ND and YF) obtained in Examples 2 to 6 were determined in the following manner. As the control, the naturally occurring KOD polymerase (Toyobo Co., Ltd.) was used. 50 µl of a reaction solution (120 mM Tris-HCl (pH 8.8 at 25 °C), 10 mM KCl, 6 mM ammonium sulfate, 1 mM $MgCl_2$, 0.1 % Triton X-100, 0.001 % BSA, 5 µg tritium-labeled E. coli DNA) was put to a 1.5 ml Eppendorf tube, and the DNA polymerase was added in amounts of 25, 50 and 100 units respectively. The naturally occurring KOD

polymerase was used in amounts of 0.25, 0.5 and 1 unit respectively. After the mixture was reacted at 75 °C for 10 minutes, the reaction was terminated by cooling on ice. Then, 50 μl of 0.1 % BSA was added as a carrier to it, and then 100 μl of a solution containing 10 % trichloroacetic acid and 2 % sodium pyrrophosphate was mixed with it. After the mixture was left for 15 minutes on ice, it is centrifuged at 12,000 r.p.m. for 10 minutes to separate the precipitates present. 100 μl of the supernatant was measured for radioactivity in a liquid scintillation counter (Packard) whereby the amount of the nucleotide delivered into the acid soluble fragment was determined.

FIG. 1 shows the polymerase activity of each DNA polymerase and the decomposition rate of DNA. In this result, the exonuclease activity of the 3 modified thermostable DNA polymerases (DEA, DA and EA) could not be detected. The modified thermostable DNA polymerase (ND) had about 0.1 %, and the modified thermostable DNA polymerase (YF) had about 0.01 % of the activity of the naturally occurring KOD polymerase.

Example 8.

Confirmation of Thermostability

The thermostability of the modified thermostable DNA polymerases obtained in Examples 2 to 6 (DA, EA, DEA, ND and YF) was determined in the following manner. 5 units of each purified modified DNA polymerase was mixed with 100 μl buffer solution (20 mM Tris-HCl pH 8.8 at 25 °C, 10 mM potassium chloride, 10 mM ammonium sulfate, 2 mM magnesium sulfate, 0.1 % Triton X-100, 0.1 mg/ml BSA, 5 mM 2-mercaptoethanol) and then pre-incubated at 95 °C. A sample was recovered from this mixture with time, and its polymerase activity was determined in the method described in Example 2.

For comparison, Taq polymerase (Toyobo Co., Ltd.) and the naturally occurring KOD polymerase (Toyobo Co., Ltd.) were also subjected to the same procedure. As shown in FIG. 2, any of the modified thermostable DNA polymerases, similar to the naturally occurring KOD polymerase, had 60 % or more residual activity after treatment at 95 °C for 6 hours. On the other hand, Taq polymerase had 15 % or less residual activity.

Example 9.

Measurement of DNA Extension Rate

The modified thermostable DNA polymerases obtained in Examples 2 to 6 (DA, EA, DEA, ND and YF) was examined for DNA extension rate in the following manner. 0.2 μg of the primer (SEQ ID NO: 15) was annealed to a single-stranded chain of M13mp18 DNA, and then 1 unit of each purified modified DNA polymerase was reacted with the single-stranded chain at 75 °C for 20, 40, and 60 seconds respectively in 10 μl of a reaction solution (20 mM Tris-HCl (pH 7.5), 8 mM magnesium chloride, 7.5 mM dithiothreitol, 100 μg/ml BSA, 0.1 mM dNTP, 0.2 μCi [α-^{32}P]dCTP). The reaction was terminated by adding an equal volume of a reaction terminating solution (50 mM sodium hydroxide, 10 mM EDTA, 5 % Ficoll, 0.05 Bromophenol Blue). For comparison, Taq polymerase (Toyobo Co., Ltd.) and the naturally occurring KOD polymerase (Toyobo Co., Ltd.) were also subjected to the same procedure.

The DNA extended by the reaction was fractionated by electrophoresis on alkali agarose gel, and the gel was dried and subjected to autoradiography. As a DNA size marker, labeled λ/HindIII was used. The DNA extension rate was determined using the size of the extended DNA determined with a band of this marker as an indicator. The result indicated that similar to the naturally occurring KOD polymerase, any of the modified polymerases had an extension rate of about 120 bases/second, while Taq polymerase had an extension rate of about 60 bases/second.

Example 10.

Preparation of Mutant Gene (IN) and Purification of Modified Thermostable DNA Polymerase

Plasmid pKOD1 obtained in Example 1 was used to prepare a plasmid (pKODIN) carrying a gene for modified thermostable DNA polymerase where in the $X_1DX_2EX_3$ motif located in the EXO1 region, isoleucine at X_2 had been replaced by asparagine.

This plasmid was prepared using a Chameleon site-directed mutagenesis kit (Stratagene) according to the manufacture's instructions.

The selective primer used was a primer as shown in SEQ ID NO: 16. The mutagenesis primer used was a primer as shown in SEQ ID NO: 17. The mutant was confirmed by determining its nucleotide sequence. E. coli JM109 was transformed with the plasmid to give JM109 (pKODIN).

Example 11.

Preparation of Mutant Gene (IE) and Purification of Modified Thermostable DNA Polymerase

A thermostable polymerase gene (pKODIE) for KOD polymerase where in the $X_1DX_2EX_3$ motif located in the EXO1 region, isoleucine at X_2 had been replaced by glutamic acid was prepared in the same manner as in Example 10.

The selective primer used was a primer as shown in SEQ ID NO: 16. The mutagenesis primer used was a primer as shown in SEQ ID NO: 18. The modified thermostable DNA polymerase (IE) was obtained using the same purification method as in Example 10.

Example 12.

Preparation of Mutant Gene (IQ) and Purification of Modified Thermostable DNA Polymerase

A thermostable polymerase gene (pKODIQ) for KOD polymerase where, in the $X_1DX_2EX_3$ motif located in the EXO1 region, isoleucine at X_2 had been replaced by glutamic acid was prepared in the same manner as in Example 10.

The selective primer used was a primer as shown in SEQ ID NO: 16. The mutagenesis primer used was a primer as shown in SEQ ID NO: 19. The modified thermostable DNA polymerase (IQ) was obtained using the same purification method as in Example 10.

Example 13.

Preparation of Mutant Gene (ID) and Purification of Modified Thermostable DNA Polymerase

A thermostable polymerase gene (pKODID) for KOD polymerase where, in the $X_1DX_2EX_3$ motif located in the EXO1 region, isoleucine at X_2 had been replaced by aspartic acid was prepared in the same manner as in Example 10.

The selective primer used was a primer as shown in SEQ ID NO: 16. The mutagenesis primer used was a primer as shown in SEQ ID NO: 20. The modified thermostable DNA polymerase (ID) was obtained using the same purification method as in Example 10.

Example 14.

Preparation of Mutant Gene (TV) and Purification of Modified Thermostable DNA Polymerase

A thermostable polymerase gene (pKODTV) for KOD polymerase where, in the $X_1DX_2EX_3$ motif located in the EXO1 region, tyrosine at X_3 had been replaced by valine was prepared in the same manner as in Example 10.

The selective primer used was a primer as shown in SEQ ID NO: 16. The mutagenesis primer used was a primer as shown in SEQ ID NO: 21. The modified thermostable DNA polymerase (TV) was obtained using the same purification method as in Example 10.

Example 15.

Preparation of Mutant Gene (IK) and Purification of Modified Thermostable DNA Polymerase

A thermostable polymerase gene (pKODIK) for KOD polymerase where, in the $X_1DX_2EX_3$ motif located in the EXO1 region, isoleucine at X_2 had been replaced by lysin was prepared in the same manner as in Example 10.

The selective primer used was a primer as shown in SEQ ID NO: 16. The mutagenesis primer used was a primer as shown in SEQ ID NO: 23. The modified thermostable DNA polymerase (IK) was obtained using the same purification method as in Example 10.

Example 16.

Preparation of Mutant Gene (IR) and Purification of Modified Thermostable DNA Polymerase

A thermostable polymerase gene (pKODIR) for KOD polymerase where, in the $X_1DX_2EX_3$ motif located in the EXO1 region, isoleucine at X_2 had been replaced by arginine was prepared in the same manner as in Example 10.

The selective primer used was a primer as shown in SEQ ID NO: 16. The mutagenesis primer used was a primer as shown in SEQ ID NO: 22. The modified thermostable DNA polymerase (IR) was obtained using the same purification

method as in Example 10.

Example 17.

Confirmation of Exonuclease Activity of Modified Thermostable DNA Polymerase

The modified thermostable DNA polymerases obtained in Examples 10 to 16 (IN, IE, IQ, ID, YV, IK and IR) were examined for exonuclease activity in the following manner. As the control, the naturally occurring KOD polymerase (Toyobo Co. Ltd.) was used. 50 μl of a reaction solution (120 mM Tris-HCl (pH 8.8 at 25 °C), 10 mM KCl, 6 mM ammonium sulfate, 1 mM $MgCl_2$, 0.1 % Triton X-100, 0.001 % BSA, 5 μg of tritium-labeled E. coli DNA) was pipetted into a 1.5-ml Eppendorf tube, followed by adding each DNA polymerase in amounts of 0.5, 1, and 1.5 units respectively. After the mixture was reacted at 75 °C for 10 minutes, the reaction was terminated on cooling on ice. Then, 50 ml of 0.1 % BSA was added to it as a carrier, and then 100 μl of a solution containing 10 % trichloroacetic acid and 2 % sodium pyrrophosphate was mixed with it. After the mixture was left on ice for 15 minutes, it was centrifuged at 12,000 r.p.m. for 10 minutes to separate the precipitates present. 100 μl of the supernatant was measured for radioactivity in a liquid scintillation counter (Packard) whereby the amount of the nucleotide delivered into the acid soluble fragment was determined.

FIG. 7 shows the polymerase activity of each DNA polymerase and the decomposition rate of DNA. FIG. 8 shows their exonuclease activities relative to that of the naturally occurring KOD polymerase. As shown therein, the thermostable DNA polymerases with the 3'-5' exonuclease activity at different levels can be obtained according to the present invention.

As compared with the naturally occurring KOD polymerase, the modified thermostable DNA polymerases had the 3'-5' exonuclease activity at the following levels: IN had about 95 %; IE, about 76 %; IQ, about 64 %; ID, about 52 %; TV, about 48 %; IK, about 30 %; and IR, about 0 %.

Example 18.

Measurement of Fidelity of DNA Extension in PCR by Modified DNA Polymerase

The naturally occurring KOD polymerase, the modified thermostable DNA polymerases IE, ID, IK and IR, and Taq polymerase were examined for fidelity of DNA extension in PCR, as follows:

Plasmid pUR288 (described in Current Protocols in Molecular Biology 1.5.6) was cleaved with restriction enzyme ScaI. PCR was conducted using 1 ng of this plasmid and the primers of SEQ ID NOS: 13 and 14. After the reaction was finished, 5 μl of the reaction solution was subjected to agarose gel electrophoresis, and amplification of the about 5.3 kb target was confirmed. The remainder of the reaction solution was treated with phenol/chloroform and then precipitated with ethanol. The precipitate was dried and dissolved in 50 μl High buffer (50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT). Further, 10 units of restriction enzyme ScaI (Toyobo Co., Ltd.) were added to it and the mixture was reacted at 37 °C for 16 hours. The target amplification product was separated by agarose gel electrophoresis and its corresponding agarose gel was cut off from the gel. From the agarose, the target DNA was purified using Gene Clean 2 (BIO101). 10 ng of the DNA thus purified was diluted to 10 μl with TE buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA). To this solution was added 10 μl of a reaction solution from a ligation kit (Ligation high, Toyobo Co., Ltd.), and the mixture was reacted at 16 °C for 30 minutes. Then, the resulting DNA was transformed into commercially available competent cells (competent high JM109, Toyobo K.K.).

The transformant was cultured at 35 °C for 16 hours in an LB agar medium (1 % Bacto-trypton, 0.5 % yeast extract, 0.5 % sodium chloride, 1.5 % agar, produced by Gibco) containing 100 μg/ml ampicillin, 1mM isopropylthio-β-galactoside (IPTG, Nakarai Tesque), 0.7 % 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal (Nakarai Tesque)) and then their colonies were counted. pUR288 carries the lacZ gene (β-D-galactosidase). Therefore, if DNA extension has proceeded with fidelity during PCR, blue colonies are formed on the agar medium. On the contrary, if misincorporation has occurred during DNA extension, the activity of β-galactosidase encoded by the lacZ gene is reduced or lost, resulting in occurrence of pale blue or white colonies. Assuming these plate blue colonies and white colonies are mutant colonies, mutant frequency (%) was determined when each enzyme was used, and the results are shown in Table 1 below.

Table 1

	KOD	IE	ID	IK	IE	rTaq
Colonies in Total	2394	3267	4869	2826	1197	2831
Mutant Colonies	19	63	148	362	299	795
Mutant Frequency (%)	0.79	1.9	3.0	12.8	25.0	28.1

As is evident from Table 1, the modified thermostable DNA polymerases IE, ID, IK and IR obtained in the present invention were inferior to the naturally occurring KOD polymerase, but they showed lower degrees of mutation than that of Taq polymerase, that is, they demonstrated higher fidelity in DNA extension.

Example 19.

PCR by Use of Modified DNA Polymerase (for Plasmid)

PCR was carried out using naturally occurring KOD polymerase (described in Unexamined Published Japanese Patent Application No. 298,879/1995) and the modified the rmostable DNA polymerase (described in Example 5), as follows: 2.5 units of each enzyme were added to 50 μl of a reaction solution (120 mM Tris-HCl (pH 8.0 at 25 °C), 10 mM KCl, 6 mM ammonium sulfate, 1 mM $MgCl_2$, 0.2 mM dNTP, 0.1 % Triton X-100, 0.001 % BSA, 1 ng plasmid pBR322 rendered linear with restriction enzyme ScaI, and 10 pmol primers shown in SEQ ID NOS: 13 and 14), and PCR was carried out. The thermal cycler used was Model PJ2000 (Perkin Elmer). The reaction conditions were 94 °C, 30 seconds → 68 °C, 2.5 minutes, and this cycle was repeated 25 times. Taq polymerase (Toyobo K.K.) was subjected to PCR in the same manner except that the reaction solution was 10 mM Tris-HCl (pH 8.8 at 25 °C) containing 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.1 % Triton X-100, 1 ng plasmid pBR322 rendered linear with restriction enzyme ScaI, and 10 pmol primers shown in SEQ ID NOS: 13 and 14. After the reaction was finished, 5 μl of the reaction solution was subjected to agarose gel electrophoresis, and amplification of the about 4.3 kb target was confirmed.

FIG. 3 shows the result of agarose gel electrophoresis. This result indicated that PCR amplification by the modified DNA polymerase was better than that by the naturally occurring KOD polymerase. Further, this amplification was better than that by Taq polymerase.

Example 20.

PCR by Use of Modified DNA Polymerase (for Human Genome)

PCR was carried out using the modified thermostable DNA polymerase (described in Example 5) as follows: 2.5 units of the enzyme were added to 50 μl of a reaction solution (120 mM Tris-HCl (pH 8.0 at 25 °C), 10 mM KCl, 6 mM ammonium sulfate, 1 mM $MgCl_2$, 0.2 mM dNTP, 0.1 % Triton X-100, 0.001 % BSA, 100 ng genomic DNA (Clontech) derived from human placenta, and 10 pmol primers shown in SEQ ID NOS: 11 and 12), and PCR was carried out. The thermal cycler used was Model PJ2000 (Perkin Elmer). The reaction conditions were 94 °C, 30 seconds → 68 °C, 3 minutes, and this cycle was repeated 25 times.

For comparison, Taq polymerase (Toyobo K.K.) was also subjected to PCR in the same manner except that the reaction solution was 10 mM Tris-HCl (pH 8.8 at 25 °C) containing 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.1 % Triton X-100, 100 ng genomic DNA (Clontech) derived from human placenta, and 10 pmol primers shown in SEQ ID NOS: 11 and 12. After the reaction was finished, 5 μl of the reaction solution was subjected to agarose gel electrophoresis, and amplification of the about 4 kb target was confirmed. FIG. 4 shows the result of agarose gel electrophoresis. This result indicated that PCR amplification by the modified DNA polymerase was better than that by Taq polymerase.

Example 21.

PCR by Use of DNA Polymerase Composition (for Human Genome)

PCR was carried out using a mixture of the modified thermostable DNA polymerase (DA, EA, DEA, ND or YF) and naturally occurring KOD polymerase, as follows: 2.5 units of ND and 0.05 unit of KOD polymerase were added to 50 μl of a reaction solution (120 mM Tris-HCl (pH 8.8 at 25 °C), 10 mM KCl, 6 mM ammonium sulfate, 1 mM $MgCl_2$, 0.2 mM dNTP, 0.1 % Triton X-100, 0.001 % BSA, 30 ng genomic DNA (Clontech) derived from human placenta, and 10 pmol

primers shown in SEQ ID NOS: 11 and 12). The thermal cycler used was Model PJ2000 (Perkin Elmer). The reaction conditions were 94 °C, 30 seconds → 68 °C, 3 minutes, and this cycle was repeated 30 times.

For comparison, the modified thermostable DNA polymerase (ND), Taq polymerase (Toyobo Co., Ltd.), a commercial DNA polymerase mixture (ExTaq (Takara Shuzo Co., Ltd.), and Advantage Tth (Clontech) were subjected respectively to PCR using the same amounts of the genomic DNA and primers in the same manner except that the reaction solution was the buffer attached to the commercial product. After the reaction was finished, 5 μl of the reaction solution was subjected to agarose gel electrophoresis, and amplification of the about 4 kb target was confirmed. FIG. 5 shows the result of agarose gel electrophoresis. This result indicated that PCR amplification by a mixture of the modified DNA polymerase (ND) and the naturally occurring KOD polymerase was better than that by the commercial polymerase mixture.

Nucleic acid amplification excellent in efficiency of amplification can be effected by a mixture of 2 or more DNA polymerases which are almost identical to each other with respect to thermostability and DNA extension rate but are different in their 3'-5' exonuclease activity.

SEQUENCE LISTING

SEQ. ID NO: 1

LENGTH: 5342 base pairs

TYPE: nucleic acid (DNA)

STRANDEDNESS: double

TOPOLOGY: linear

MOLECULAR TYPE: genomic DNA

SOURCE: hyperthermophilic archaeon

STRAIN NAME: KOD1

CHARACTERISTICS:

156-5165 P CDS

1374-2453 intervening sequence

2708-4316 intervening sequence

SEQUENCE DESCRIPTION:

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GCTTGAGGGC CTGCGGTTAT GGGACGTTGC AGTTTGCGCC TACTCAAAGA TGCCGGTTTT   60
ATAACGGAGA AAAATGGGGA GCTATTACGA TCTCTCCTTG ATGTGGGGTT TACAATAAAG  120
CCTGGATTGT TCTACAAGAT TATGGGGGAT GAAAG ATG ATC CTC GAC ACT GAC      173
                                       Met Ile Leu Asp Thr Asp
                                        1               5
TAC ATA ACC GAG GAT GGA AAG CCT GTC ATA AGA ATT TTC AAG AAG GAA     221
Tyr Ile Thr Glu Asp Gly Lys Pro Val Ile Arg Ile Phe Lys Lys Glu
            10                  15                  20
AAC GGC GAG TTT AAG ATT GAG TAC GAC CGG ACT TTT GAA CCC TAC TTC     269
Asn Gly Glu Phe Lys Ile Glu Tyr Asp Arg Thr Phe Glu Pro Tyr Phe
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                25                  30                  35
TAC GCC CTC CTG AAG GAC GAT TCT GCC ATT GAG GAA GTC AAG AAG ATA    317
Tyr Ala Leu Leu Lys Asp Asp Ser Ala Ile Glu Glu Val Lys Lys Ile
        40                  45                  50
ACC GCC GAG AGG CAC GGG ACG GTT GTA ACG GTT AAG CGG GTT GAA AAG    365
Thr Ala Glu Arg His Gly Thr Val Val Thr Val Lys Arg Val Glu Lys
    55                  60                  65                  70
GTT CAG AAG AAG TTC CTC GGG AGA CCA GTT GAG GTC TGG AAA CTC TAC    413
Val Gln Lys Lys Phe Leu Gly Arg Pro Val Glu Val Trp Lys Leu Tyr
                75                  80                  85
TTT ACT CAT CCG CAG GAC GTC CCA GCG ATA AGG GAC AAG ATA CGA GAG    461
Phe Thr His Pro Gln Asp Val Pro Ala Ile Arg Asp Lys Ile Arg Glu
            90                  95                  100
CAT GGA GCA GTT ATT GAC ATC TAC GAG TAC GAC ATA CCC TTC GCC AAG    509
His Gly Ala Val Ile Asp Ile Tyr Glu Tyr Asp Ile Pro Phe Ala Lys
        105                 110                 115
CGC TAC CTC ATA GAC AAG GGA TTA GTG CCA ATG GAA GGC GAC GAG GAG    557
Arg Tyr Leu Ile Asp Lys Gly Leu Val Pro Met Glu Gly Asp Glu Glu
    120                 125                 130
CTG AAA ATG CTC GCC TTC GAC ATT GAA ACT CTC TAC CAT GAG GGC GAG    605
Leu Lys Met Leu Ala Phe Asp Ile Glu Thr Leu Tyr His Glu Gly Glu
135                 140                 145                 150
GAG TTC GCC GAG GGG CCA ATC CTT ATG ATA AGC TAC GCC GAC GAG GAA    653
Glu Phe Ala Glu Gly Pro Ile Leu Met Ile Ser Tyr Ala Asp Glu Glu
                155                 160                 165
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GGG GCC AGG GTG ATA ACT TGG AAG AAC GTG GAT CTC CCC TAC GTT GAC    701
Gly Ala Arg Val Ile Thr Trp Lys Asn Val Asp Leu Pro Tyr Val Asp
            170                 175                 180

GTC GTC TCG ACG GAG AGG GAG ATG ATA AAG CGC TTC CTC CGT GTT GTG    749
Val Val Ser Thr Glu Arg Glu Met Ile Lys Arg Phe Leu Arg Val Val
        185                 190                 195

AAG GAG AAA GAC CCG GAC GTT CTC ATA ACC TAC AAC GGC GAC AAC TTC    797
Lys Glu Lys Asp Pro Asp Val Leu Ile Thr Tyr Asn Gly Asp Asn Phe
    200                 205                 210

GAC TTC GCC TAT CTG AAA AAG CGC TGT GAA AAG CTC GGA ATA AAC TTC    845
Asp Phe Ala Tyr Leu Lys Lys Arg Cys Glu Lys Leu Gly Ile Asn Phe
215                 220                 225                 230

GCC CTC GGA AGG GAT GGA AGC GAG CCG AAG ATT CAG AGG ATG GGC GAC    893
Ala Leu Gly Arg Asp Gly Ser Glu Pro Lys Ile Gln Arg Met Gly Asp
                235                 240                 245

AGG TTT GCC GTC GAA GTG AAG GGA CGG ATA CAC TTC GAT CTC TAT CCT    941
Arg Phe Ala Val Glu Val Lys Gly Arg Ile His Phe Asp Leu Tyr Pro
            250                 255                 260

GTG ATA AGA CGG ACG ATA AAC CTG CCC ACA TAC ACG CTT GAG GCC GTT    989
Val Ile Arg Arg Thr Ile Asn Leu Pro Thr Tyr Thr Leu Glu Ala Val
        265                 270                 275

TAT GAA GCC GTC TTC GGT CAG CCG AAG GAG AAG GTT TAC GCT GAG GAA   1037
Tyr Glu Ala Val Phe Gly Gln Pro Lys Glu Lys Val Tyr Ala Glu Glu
    280                 285                 290

ATA ACA CCA GCC TGG GAA ACC GGC GAG AAC CTT GAG AGA GTC GCC CGC   1085
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Ile Thr Pro Ala Trp Glu Thr Gly Glu Asn Leu Glu Arg Val Ala Arg

295 300 305 310

TAC TCG ATG GAA GAT GCG AAG GTC ACA TAC GAG CTT GGG AAG GAG TTC 1133

Tyr Ser Met Glu Asp Ala Lys Val Thr Tyr Glu Leu Gly Lys Glu Phe

315 320 325

CTT CCG ATG GAG GCC CAG CTT TCT CGC TTA ATC GGC CAG TCC CTC TGG 1181

Leu Pro Met Glu Ala Gln Leu Ser Arg Leu Ile Gly Gln Ser Leu Trp

330 335 340

GAC GTC TCC CGC TCC AGC ACT GGC AAC CTC GTT GAG TGG TTC CTC CTC 1229

Asp Val Ser Arg Ser Ser Thr Gly Asn Leu Val Glu Trp Phe Leu Leu

345 350 355

AGG AAG GCC TAT GAG AGG AAT GAG CTG GCC CCG AAC AAG CCC GAT GAA 1277

Arg Lys Ala Tyr Glu Arg Asn Glu Leu Ala Pro Asn Lys Pro Asp Glu

360 365 370

AAG GAG CTG GCC AGA AGA CGG CAG AGC TAT GAA GGA GGC TAT GTA AAA 1325

Lys Glu Leu Ala Arg Arg Arg Gln Ser Tyr Glu Gly Gly Tyr Val Lys

375 380 385 390

GAG CCC GAG AGA GGG TTG TGG GAG AAC ATA GTG TAC CTA GAT TTT AGA 1373

Glu Pro Glu Arg Gly Leu Trp Glu Asn Ile Val Tyr Leu Asp Phe Arg

395 400 405

TGC CAT CCA GCC GAT ACG AAG GTT GTC GTC AAG GGG AAG GGG ATT ATA 1421

Cys His Pro Ala Asp Thr Lys Val Val Val Lys Gly Lys Gly Ile Ile

410 415 420

AAC ATC AGC GAG GTT CAG GAA GGT GAC TAT GTC CTT GGG ATT GAC GGC 1469

Asn Ile Ser Glu Val Gln Glu Gly Asp Tyr Val Leu Gly Ile Asp Gly

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                425                 430                 435
TGG CAG AGA GTT AGA AAA GTA TGG GAA TAC GAC TAC AAA GGG GAG CTT   1517
Trp Gln Arg Val Arg Lys Val Trp Glu Tyr Asp Tyr Lys Gly Glu Leu
    440                 445                 450
GTA AAC ATA AAC GGG TTA AAG TGT ACG CCC AAT CAT AAG CTT CCC GTT   1565
Val Asn Ile Asn Gly Leu Lys Cys Thr Pro Asn His Lys Leu Pro Val
455                 460                 465                 470
GTT ACA AAG AAC GAA CGA CAA ACG AGA ATA AGA GAC AGT CTT GCT AAG   1613
Val Thr Lys Asn Glu Arg Gln Thr Arg Ile Arg Asp Ser Leu Ala Lys
                475                 480                 485
TCT TTC CTT ACT AAA AAA GTT AAG GGC AAG ATA ATA ACC ACT CCC CTT   1661
Ser Phe Leu Thr Lys Lys Val Lys Gly Lys Ile Ile Thr Thr Pro Leu
            490                 495                 500
TTC TAT GAA ATA GGC AGA GCG ACA AGT GAG AAT ATT CCA GAA GAA GAG   1709
Phe Tyr Glu Ile Gly Arg Ala Thr Ser Glu Asn Ile Pro Glu Glu Glu
        505                 510                 515
GTT CTC AAG GGA GAG CTC GCT GGC ATA CTA TTG GCT GAA GGA ACG CTC   1757
Val Leu Lys Gly Glu Leu Ala Gly Ile Leu Leu Ala Glu Gly Thr Leu
    520                 525                 530
TTG AGG AAA GAC GTT GAA TAC TTT GAT TCA TCC CGC AAA AAA CGG AGG   1805
Leu Arg Lys Asp Val Glu Tyr Phe Asp Ser Ser Arg Lys Lys Arg Arg
535                 540                 545                 550
ATT TCA CAC CAG TAT CGT GTT GAG ATA ACC ATT GGG AAA GAC GAG GAG   1853
Ile Ser His Gln Tyr Arg Val Glu Ile Thr Ile Gly Lys Asp Glu Glu
                555                 560                 565
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GAG TTT AGG GAT CGT ATC ACA TAC ATT TTT GAG CGT TTG TTT GGG ATT 1901

Glu Phe Arg Asp Arg Ile Thr Tyr Ile Phe Glu Arg Leu Phe Gly Ile

570 575 580

ACT CCA AGC ATC TCG GAG AAG AAA GGA ACT AAC GCA GTA ACA CTC AAA 1949

Thr Pro Ser Ile Ser Glu Lys Lys Gly Thr Asn Ala Val Thr Leu Lys

585 590 595

GTT GCG AAG AAG AAT GTT TAT CTT AAA GTC AAG GAA ATT ATG GAC AAC 1997

Val Ala Lys Lys Asn Val Tyr Leu Lys Val Lys Glu Ile Met Asp Asn

600 605 610

ATA GAG TCC CTA CAT GCC CCC TCG GTT CTC AGG GGA TTC TTC GAA GGC 2045

Ile Glu Ser Leu His Ala Pro Ser Val Leu Arg Gly Phe Phe Glu Gly

615 620 625 630

GAC GGT TCA GTA AAC AGG GTT AGG AGG AGT ATT GTT GCA ACC CAG GGT 2093

Asp Gly Ser Val Asn Arg Val Arg Arg Ser Ile Val Ala Thr Gln Gly

635 640 645

ACA AAG AAC GAG TGG AAG ATT AAA CTG GTG TCA AAA CTG CTC TCC CAG 2141

Thr Lys Asn Glu Trp Lys Ile Lys Leu Val Ser Lys Leu Leu Ser Gln

650 655 660

CTT GGT ATC CCT CAT CAA ACG TAC ACG TAT CAG TAT CAG GAA AAT GGG 2189

Leu Gly Ile Pro His Gln Thr Tyr Thr Tyr Gln Tyr Gln Glu Asn Gly

665 670 675

AAA GAT CGG AGC AGG TAT ATA CTG GAG ATA ACT GGA AAG GAC GGA TTG 2237

Lys Asp Arg Ser Arg Tyr Ile Leu Glu Ile Thr Gly Lys Asp Gly Leu

680 685 690

ATA CTG TTC CAA ACA CTC ATT GGA TTC ATC AGT GAA AGA AAG AAC GCT 2285

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Ile Leu Phe Gln Thr Leu Ile Gly Phe Ile Ser Glu Arg Lys Asn Ala
695                 700                 705                 710
CTG CTT AAT AAG GCA ATA TCT CAG AGG GAA ATG AAC AAC TTG GAA AAC  2333
Leu Leu Asn Lys Ala Ile Ser Gln Arg Glu Met Asn Asn Leu Glu Asn
                715                 720                 725
AAT GGA TTT TAC AGG CTC AGT GAA TTC AAT GTC AGC ACG GAA TAC TAT  2381
Asn Gly Phe Tyr Arg Leu Ser Glu Phe Asn Val Ser Thr Glu Tyr Tyr
            730                 735                 740
GAG GGC AAG GTC TAT GAC TTA ACT CTT GAA GGA ACT CCC TAC TAC TTT  2429
Glu Gly Lys Val Tyr Asp Leu Thr Leu Glu Gly Thr Pro Tyr Tyr Phe
        745                 750                 755
GCC AAT GGC ATA TTG ACC CAT AAC TCC CTG TAC CCC TCA ATC ATC ATC  2477
Ala Asn Gly Ile Leu Thr His Asn Ser Leu Tyr Pro Ser Ile Ile Ile
    760                 765                 770
ACC CAC AAC GTC TCG CCG GAT ACG CTC AAC AGA GAA GGA TGC AAG GAA  2525
Thr His Asn Val Ser Pro Asp Thr Leu Asn Arg Glu Gly Cys Lys Glu
775                 780                 785                 790
TAT GAC GTT GCC CCA CAG GTC GGC CAC CGC TTC TGC AAG GAC TTC CCA  2573
Tyr Asp Val Ala Pro Gln Val Gly His Arg Phe Cys Lys Asp Phe Pro
                795                 800                 805
GGA TTT ATC CCG AGC CTG CTT GGA GAC CTC CTA GAG GAG AGG CAG AAG  2621
Gly Phe Ile Pro Ser Leu Leu Gly Asp Leu Leu Glu Glu Arg Gln Lys
            810                 815                 820
ATA AAG AAG AAG ATG AAG GCC ACG ATT GAC CCG ATC GAG AGG AAG CTC  2669
Ile Lys Lys Lys Met Lys Ala Thr Ile Asp Pro Ile Glu Arg Lys Leu
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          825                 830                 835
CTC GAT TAC AGG CAG AGG GCC ATC AAG ATC CTG GCA AAC AGC ATC CTA   2717
Leu Asp Tyr Arg Gln Arg Ala Ile Lys Ile Leu Ala Asn Ser Ile Leu
    840                 845                 850
CCC GAG GAA TGG CTT CCA GTC CTC GAG GAA GGG GAG GTT CAC TTC GTC   2765
Pro Glu Glu Trp Leu Pro Val Leu Glu Glu Gly Glu Val His Phe Val
855                 860                 865                 870
AGG ATT GGA GAG CTC ATA GAC CGG ATG ATG GAG GAA AAT GCT GGG AAA   2813
Arg Ile Gly Glu Leu Ile Asp Arg Met Met Glu Glu Asn Ala Gly Lys
                875                 880                 885
GTA AAG AGA GAG GGC GAG ACG GAA GTG CTT GAG GTC AGT GGG CTT GAA   2861
Val Lys Arg Glu Gly Glu Thr Glu Val Leu Glu Val Ser Gly Leu Glu
            890                 895                 900
GTC CCG TCC TTT AAC AGG AGA ACT AAC AAG GCC GAG CTC AAG AGA GTA   2909
Val Pro Ser Phe Asn Arg Arg Thr Asn Lys Ala Glu Leu Lys Arg Val
        905                 910                 915
AAG GCC CTG ATT AGG CAC GAT TAT TCT GGC AAG GTC TAC ACC ATC AGA   2957
Lys Ala Leu Ile Arg His Asp Tyr Ser Gly Lys Val Tyr Thr Ile Arg
    920                 925                 930
CTG AAG TCG GGG AGG AGA ATA AAG ATA ACC TCT GGC CAC AGC CTC TTC   3005
Leu Lys Ser Gly Arg Arg Ile Lys Ile Thr Ser Gly His Ser Leu Phe
935                 940                 945                 950
TCT GTG AGA AAC GGG GAG CTC GTT GAA GTT ACG GGC GAT GAA CTA AAG   3053
Ser Val Arg Asn Gly Glu Leu Val Glu Val Thr Gly Asp Glu Leu Lys
                955                 960                 965
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CCA GGT GAC CTC GTT GCA GTC CCG CGG AGA TTG GAG CTT CCT GAG AGA   3101
Pro Gly Asp Leu Val Ala Val Pro Arg Arg Leu Glu Leu Pro Glu Arg
                970                 975                 980

AAC CAC GTG CTG AAC CTC GTT GAA CTG CTC CTT GGA ACG CCA GAA GAA   3149
Asn His Val Leu Asn Leu Val Glu Leu Leu Leu Gly Thr Pro Glu Glu
        985                 990                 995

GAA ACT TTG GAC ATC GTC ATG ACG ATC CCA GTC AAG GGT AAG AAG AAC   3197
Glu Thr Leu Asp Ile Val Met Thr Ile Pro Val Lys Gly Lys Lys Asn
    1000                1005                1010

TTC TTT AAA GGG ATG CTC AGG ACT TTG CGC TGG ATT TTC GGA GAG GAA   3245
Phe Phe Lys Gly Met Leu Arg Thr Leu Arg Trp Ile Phe Gly Glu Glu
1015                1020                1025                1030

AAG AGG CCC AGA ACC GCG AGA CGC TAT CTC AGG CAC CTT GAG GAT CTG   3293
Lys Arg Pro Arg Thr Ala Arg Arg Tyr Leu Arg His Leu Glu Asp Leu
                1035                1040                1045

GGC TAT GTC CGG CTT AAG AAG ATC GGC TAC GAA GTC CTC GAC TGG GAC   3341
Gly Tyr Val Arg Leu Lys Lys Ile Gly Tyr Glu Val Leu Asp Trp Asp
            1050                1055                1060

TCA CTT AAG AAC TAC AGA AGG CTC TAC GAG GCG CTT GTC GAG AAC GTC   3389
Ser Leu Lys Asn Tyr Arg Arg Leu Tyr Glu Ala Leu Val Glu Asn Val
        1065                1070                1075

AGA TAC AAC GGC AAC AAG AGG GAG TAC CTC GTT GAA TTC AAT TCC ATC   3437
Arg Tyr Asn Gly Asn Lys Arg Glu Tyr Leu Val Glu Phe Asn Ser Ile
    1080                1085                1090

CGG GAT GCA GTT GGC ATA ATG CCC CTA AAA GAG CTG AAG GAG TGG AAG   3485
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Arg Asp Ala Val Gly Ile Met Pro Leu Lys Glu Leu Lys Glu Trp Lys
1095                1100                1105                1110
ATC GGC ACG CTG AAC GGC TTC AGA ATG AGA AAG CTC ATT GAA GTG GAC   3533
Ile Gly Thr Leu Asn Gly Phe Arg Met Arg Lys Leu Ile Glu Val Asp
                1115                1120                1125
GAG TCG TTA GCA AAG CTC CTC GGC TAC TAC GTG AGC GAG GGC TAT GCA   3581
Glu Ser Leu Ala Lys Leu Leu Gly Tyr Tyr Val Ser Glu Gly Tyr Ala
            1130                1135                1140
AGA AAG CAG AGG AAT CCC AAA AAC GGC TGG AGC TAC AGC GTG AAG CTC   3629
Arg Lys Gln Arg Asn Pro Lys Asn Gly Trp Ser Tyr Ser Val Lys Leu
        1145                1150                1155
TAC AAC GAA GAC CCT GAA GTG CTG GAC GAT ATG GAG AGA CTC GCC AGC   3677
Tyr Asn Glu Asp Pro Glu Val Leu Asp Asp Met Glu Arg Leu Ala Ser
    1160                1165                1170
AGG TTT TTC GGG AAG GTG AGG CGG GGC AGG AAC TAC GTT GAG ATA CCG   3725
Arg Phe Phe Gly Lys Val Arg Arg Gly Arg Asn Tyr Val Glu Ile Pro
1175                1180                1185                1190
AAG AAG ATC GGC TAC CTG CTC TTT GAG AAC ATG TGC GGT GTC CTA GCG   3773
Lys Lys Ile Gly Tyr Leu Leu Phe Glu Asn Met Cys Gly Val Leu Ala
                1195                1200                1205
GaG AAC AAG AGG ATT CCC GAG TTC GTC TTC ACG TCC CCG AAA GGG GTT   3821
Glu Asn Lys Arg Ile Pro Glu Phe Val Phe Thr Ser Pro Lys Gly Val
            1210                1215                1220
(GG CTG GCC TTC CTT GAG GGG TAC TCA TCG GCG ATG GCG ACG TCC ACC   3869
ırg Leu Ala Phe Leu Glu Gly Tyr Ser Ser Ala Met Ala Thr Ser Thr
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                1225                1230                1235

GAA CAA GAG ACT CAG GCT CTC AAC GAA AAG CGA GCT TTA GCG AAC CAG   3917
Glu Gln Glu Thr Gln Ala Leu Asn Glu Lys Arg Ala Leu Ala Asn Gln

    1240                1245                1250

CTC GTC CTC CTC TTG AAC TCG GTG GGG GTC TCT GCT GTA AAA CTT GGG   3965
Leu Val Leu Leu Leu Asn Ser Val Gly Val Ser Ala Val Lys Leu Gly

1255                1260                1265                1270

CAC GAC AGC GGC GTT TAC AGG GTC TAT ATA AAC GAG GAG CTC CCG TTC   4013
His Asp Ser Gly Val Tyr Arg Val Tyr Ile Asn Glu Glu Leu Pro Phe

                1275                1280                1285

GTA AAG CTG GAC AAG AAA AAG AAC GCC TAC TAC TCA CAC GTG ATC CCC   4061
Val Lys Leu Asp Lys Lys Lys Asn Ala Tyr Tyr Ser His Val Ile Pro

            1290                1295                1300

AAG GAA GTC CTG AGC GAG GTC TTT GGG AAG GTT TTC CAG AAA AAC GTC   4109
Lys Glu Val Leu Ser Glu Val Phe Gly Lys Val Phe Gln Lys Asn Val

        1305                1310                1315

AGT CCT CAG ACC TTC AGG AAG ATG GTC GAG GAC GGA AGA CTC GAT CCC   4157
Ser Pro Gln Thr Phe Arg Lys Met Val Glu Asp Gly Arg Leu Asp Pro

    1320                1325                1330

GAA AAG GCC CAG AGG CTC TCC TGG CTC ATT GAG GGG GAC GTA GTG CTC   4205
Glu Lys Ala Gln Arg Leu Ser Trp Leu Ile Glu Gly Asp Val Val Leu

1335                1340                1345                1350

GAC CGC GTT GAG TCC GTT GAT GTG GAA GAC TAC GAT GGT TAT GTC TAT   4253
Asp Arg Val Glu Ser Val Asp Val Glu Asp Tyr Asp Gly Tyr Val Tyr

                1355                1360                1365
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GAC CTG AGC GTC GAG GAC AAC GAG AAC TTC CTC GTT GGC TTT GGG TTG 4301

Asp Leu Ser Val Glu Asp Asn Glu Asn Phe Leu Val Gly Phe Gly Leu

1370 1375 1380

GTC TAT GCT CAC AAC AGC TAC TAC GGT TAC TAC GGC TAT GCA AGG GCG 4349

Val Tyr Ala His Asn Ser Tyr Tyr Gly Tyr Tyr Gly Tyr Ala Arg Ala

1385 1390 1395

CGC TGG TAC TGC AAG GAG TGT GCA GAG AGC GTA ACG GCC TGG GGA AGG 4397

Arg Trp Tyr Cys Lys Glu Cys Ala Glu Ser Val Thr Ala Trp Gly Arg

1400 1405 1410

GAG TAC ATA ACG ATG ACC ATC AAG GAG ATA GAG GAA AAG TAC GGC TTT 4445

Glu Tyr Ile Thr Met Thr Ile Lys Glu Ile Glu Glu Lys Tyr Gly Phe

1415 1420 1425 1430

AAG GTA ATC TAC AGC GAC ACC GAC GGA TTT TTT GCC ACA ATA CCT GGA 4493

Lys Val Ile Tyr Ser Asp Thr Asp Gly Phe Phe Ala Thr Ile Pro Gly

1435 1440 1445

GCC GAT GCT GAA ACC GTC AAA AAG AAG GCT ATG GAG TTC CTC AAC TAT 4541

Ala Asp Ala Glu Thr Val Lys Lys Lys Ala Met Glu Phe Leu Asn Tyr

1450 1455 1460

ATC AAC GCC AAA CTT CCG GGC GCG CTT GAG CTC GAG TAC GAG GGC TTC 4589

Ile Asn Ala Lys Leu Pro Gly Ala Leu Glu Leu Glu Tyr Glu Gly Phe

1465 1470 1475

TAC AAA CGC GGC TTC TTC GTC ACG AAG AAG AAG TAT GCG GTG ATA GAC 4637

Tyr Lys Arg Gly Phe Phe Val Thr Lys Lys Lys Tyr Ala Val Ile Asp

1480 1485 1490

GAG GAA GGC AAG ATA ACA ACG CGC GGA CTT GAG ATT GTG AGG CGT GAC 4685

Glu Glu Gly Lys Ile Thr Thr Arg Gly Leu Glu Ile Val Arg Arg Asp

1495 1500 1505 1510

TGG AGC GAG ATA GCG AAA GAG ACG CAG GCG AGG GTT CTT GAA GCT TTG 4733

Trp Ser Glu Ile Ala Lys Glu Thr Gln Ala Arg Val Leu Glu Ala Leu

1515 1520 1525

CTA AAG GAC GGT GAC GTC GAG AAG GCC GTG AGG ATA GTC AAA GAA GTT 4781

Leu Lys Asp Gly Asp Val Glu Lys Ala Val Arg Ile Val Lys Glu Val

1530 1535 1540

ACC GAA AAG CTG AGC AAG TAC GAG GTT CCG CCG GAG AAG CTG GTG ATC 4829

Thr Glu Lys Leu Ser Lys Tyr Glu Val Pro Pro Glu Lys Leu Val Ile

1545 1550 1555

CAC GAG CAG ATA ACG AGG GAT TTA AAG GAC TAC AAG GCA ACC GGT CCC 4877

His Glu Gln Ile Thr Arg Asp Leu Lys Asp Tyr Lys Ala Thr Gly Pro

1560 1565 1570

CAC GTT GCC GTT GCC AAG AGG TTG GCC GCG AGA GGA GTC AAA ATA CGC 4925

His Val Ala Val Ala Lys Arg Leu Ala Ala Arg Gly Val Lys Ile Arg

1575 1580 1585 1590

CCT GGA ACG GTG ATA AGC TAC ATC GTG CTC AAG GGC TCT GGG AGG ATA 4973

Pro Gly Thr Val Ile Ser Tyr Ile Val Leu Lys Gly Ser Gly Arg Ile

1595 1600 1605

GGC GAC AGG GCG ATA CCG TTC GAC GAG TTC GAC CCG ACG AAG CAC AAG 5021

Gly Asp Arg Ala Ile Pro Phe Asp Glu Phe Asp Pro Thr Lys His Lys

1610 1615 1620

TAC GAC GCC GAG TAC TAC ATT GAG AAC CAG GTT CTC CCA GCC GTT GAG 5069

Tyr Asp Ala Glu Tyr Tyr Ile Glu Asn Gln Val Leu Pro Ala Val Glu

1625 1630 1635

AGA ATT CTG AGA GCC TTC GGT TAC CGC AAG GAA GAC CTG CGC TAC CAG 5117

Arg Ile Leu Arg Ala Phe Gly Tyr Arg Lys Glu Asp Leu Arg Tyr Gln

1640 1645 1650

AAG ACG AGA CAG GTT GGT TTG AGT GCT TGG CTG AAG CCG AAG GGA ACT 5165

Lys Thr Arg Gln Val Gly Leu Ser Ala Trp Leu Lys Pro Lys Gly Thr

1655 1660 1665 1670

TGACCTTTCC ATTTGTTTTC CAGCGGATAA CCCTTTAACT TCCCTTTCAA AAACTCCCTT 5225

TAGGGAAAGA CCATGAAGAT AGAAATCCGG CGGCGCCCGG TTAAATACGC TAGGATAGAA 5285

GTGAAGCCAG ACGGCAGGGT AGTCGTCACT GCCCCGAGGG TTCAACGTTG AGAAGTT 5342

SEQ. ID NO: 2

LENGTH: 774 amino acids

TYPE: amino acid

TOPOLOGY: linear

MOLECULAR TYPE: protein

SEQUENCE DESCRIPTION:

Met Ile Leu Asp Thr Asp Tyr Ile Thr Glu Asp Gly Lys Pro Val Ile

1 5 10 15

Arg Ile Phe Lys Lys Glu Asn Gly Glu Phe Lys Ile Glu Tyr Asp Arg

20 25 30

Thr Phe Glu Pro Tyr Phe Tyr Ala Leu Leu Lys Asp Asp Ser Ala Ile

35 40 45

Glu Glu Val Lys Lys Ile Thr Ala Glu Arg His Gly Thr Val Val Thr

50 55 60

Val Lys Arg Val Glu Lys Val Gln Lys Lys Phe Leu Gly Arg Pro Val

65 70 75 80

Glu Val Trp Lys Leu Tyr Phe Thr His Pro Gln Asp Val Pro Ala Ile

85 90 95

Arg Asp Lys Ile Arg Glu His Gly Ala Val Ile Asp Ile Tyr Glu Tyr

100 105 110

Asp Ile Pro Phe Ala Lys Arg Tyr Leu Ile Asp Lys Gly Leu Val Pro

115 120 125

Met Glu Gly Asp Glu Glu Leu Lys Met Leu Ala Phe Asp Ile Glu Thr

130 135 140

Leu Tyr His Glu Gly Glu Glu Phe Ala Glu Gly Pro Ile Leu Met Ile

145 150 155 160

Ser Tyr Ala Asp Glu Glu Gly Ala Arg Val Ile Thr Trp Lys Asn Val

165 170 175

Asp Leu Pro Tyr Val Asp Val Val Ser Thr Glu Arg Glu Met Ile Lys

180 185 190

Arg Phe Leu Arg Val Val Lys Glu Lys Asp Pro Asp Val Leu Ile Thr

195 200 205

Tyr Asn Gly Asp Asn Phe Asp Phe Ala Tyr Leu Lys Lys Arg Cys Glu

210 215 220

Lys Leu Gly Ile Asn Phe Ala Leu Gly Arg Asp Gly Ser Glu Pro Lys

225 230 235 240

Ile Gln Arg Met Gly Asp Arg Phe Ala Val Glu Val Lys Gly Arg Ile

245 250 255

His Phe Asp Leu Tyr Pro Val Ile Arg Arg Thr Ile Asn Leu Pro Thr
260 265 270

Tyr Thr Leu Glu Ala Val Tyr Glu Ala Val Phe Gly Gln Pro Lys Glu
275 280 285

Lys Val Tyr Ala Glu Glu Ile Thr Pro Ala Trp Glu Thr Gly Glu Asn
290 295 300

Leu Glu Arg Val Ala Arg Tyr Ser Met Glu Asp Ala Lys Val Thr Tyr
305 310 315 320

Glu Leu Gly Lys Glu Phe Leu Pro Met Glu Ala Gln Leu Ser Arg Leu
325 330 335

Ile Gly Gln Ser Leu Trp Asp Val Ser Arg Ser Ser Thr Gly Asn Leu
340 345 350

Val Glu Trp Phe Leu Leu Arg Lys Ala Tyr Glu Arg Asn Glu Leu Ala
355 360 365

Pro Asn Lys Pro Asp Glu Lys Glu Leu Ala Arg Arg Arg Gln Ser Tyr
370 375 380

Glu Gly Gly Tyr Val Lys Glu Pro Glu Arg Gly Leu Trp Glu Asn Ile
385 390 395 400

Val Tyr Leu Asp Phe Arg Ser Leu Tyr Pro Ser Ile Ile Ile Thr His
405 410 415

Asn Val Ser Pro Asp Thr Leu Asn Arg Glu Gly Cys Lys Glu Tyr Asp
420 425 430

Val Ala Pro Gln Val Gly His Arg Phe Cys Lys Asp Phe Pro Gly Phe
435 440 445

Ile Pro Ser Leu Leu Gly Asp Leu Leu Glu Glu Arg Gln Lys Ile Lys

450 455 460

Lys Lys Met Lys Ala Thr Ile Asp Pro Ile Glu Arg Lys Leu Leu Asp

465 470 475 480

Tyr Arg Gln Arg Ala Ile Lys Ile Leu Ala Asn Ser Tyr Tyr Gly Tyr

485 490 495

Tyr Gly Tyr Ala Arg Ala Arg Trp Tyr Cys Lys Glu Cys Ala Glu Ser

500 505 510

Val Thr Ala Trp Gly Arg Glu Tyr Ile Thr Met Thr Ile Lys Glu Ile

515 520 525

Glu Glu Lys Tyr Gly Phe Lys Val Ile Tyr Ser Asp Thr Asp Gly Phe

530 535 540

Phe Ala Thr Ile Pro Gly Ala Asp Ala Glu Thr Val Lys Lys Lys Ala

545 550 555 560

Met Glu Phe Leu Asn Tyr Ile Asn Ala Lys Leu Pro Gly Ala Leu Glu

565 570 575

Leu Glu Tyr Glu Gly Phe Tyr Lys Arg Gly Phe Phe Val Thr Lys Lys

580 585 590

Lys Tyr Ala Val Ile Asp Glu Glu Gly Lys Ile Thr Thr Arg Gly Leu

595 600 605

Glu Ile Val Arg Arg Asp Trp Ser Glu Ile Ala Lys Glu Thr Gln Ala

610 615 620

Arg Val Leu Glu Ala Leu Leu Lys Asp Gly Asp Val Glu Lys Ala Val

625 630 635 640

Arg Ile Val Lys Glu Val Thr Glu Lys Leu Ser Lys Tyr Glu Val Pro

645 650 655

Pro Glu Lys Leu Val Ile His Glu Gln Ile Thr Arg Asp Leu Lys Asp
660 665 670

Tyr Lys Ala Thr Gly Pro His Val Ala Val Ala Lys Arg Leu Ala Ala
675 680 685

Arg Gly Val Lys Ile Arg Pro Gly Thr Val Ile Ser Tyr Ile Val Leu
690 695 700

Lys Gly Ser Gly Arg Ile Gly Asp Arg Ala Ile Pro Phe Asp Glu Phe
705 710 715 720

Asp Pro Thr Lys His Lys Tyr Asp Ala Glu Tyr Tyr Ile Glu Asn Gln
725 730 735

Val Leu Pro Ala Val Glu Arg Ile Leu Arg Ala Phe Gly Tyr Arg Lys
740 745 750

Glu Asp Leu Arg Tyr Gln Lys Thr Arg Gln Val Gly Leu Ser Ala Trp
755 760 765

Leu Lys Pro Lys Gly Thr
770

SEQ. ID NO: 3

LENGTH: 2325 base pairs

TYPE: nucleic acid (DNA)

STRANDEDNESS: double

TOPOLOGY: linear

MOLECULAR TYPE: genomic DNA

SEQUENCE DESCRIPTION:

ATGATCCTCG ACACTGACTA CATAACCGAG GATGGAAAGC CTGTCATAAG AATTTTCAAG 60

AAGGAAAACG GCGAGTTTAA GATTGAGTAC GACCGGACTT TTGAACCCTA CTTCTACGCC 120

CTCCTGAAGG ACGATTCTGC CATTGAGGAA GTCAAGAAGA TAACCGCCGA GAGGCACGGG 180

ACGGTTGTAA CGGTTAAGCG GGTTGAAAAG GTTCAGAAGA AGTTCCTCGG GAGACCAGTT 240

GAGGTCTGGA AACTCTACTT TACTCATCCG CAGGACGTCC CAGCGATAAG GGACAAGATA 300

CGAGAGCATG GAGCAGTTAT TGACATCTAC GAGTACGACA TACCCTTCGC CAAGCGCTAC 360

CTCATAGACA AGGGATTAGT GCCAATGGAA GGCGACGAGG AGCTGAAAAT GCTCGCCTTC 420

GACATTGAAA CTCTCTACCA TGAGGGCGAG GAGTTCGCCG AGGGGCCAAT CCTTATGATA 480

AGCTACGCCG ACGAGGAAGG GGCCAGGGTG ATAACTTGGA AGAACGTGGA TCTCCCCTAC 540

GTTGACGTCG TCTCGACGGA GAGGGAGATG ATAAAGCGCT TCCTCCGTGT TGTGAAGGAG 600

AAAGACCCGG ACGTTCTCAT AACCTACAAC GGCGACAACT TCGACTTCGC CTATCTGAAA 660

AAGCGCTGTG AAAAGCTCGG AATAAACTTC GCCCTCGGAA GGGATGGAAG CGAGCCGAAG 720

ATTCAGAGGA TGGGCGACAG GTTTGCCGTC GAAGTGAAGG GACGGATACA CTTCGATCTC 780

TATCCTGTGA TAAGACGGAC GATAAACCTG CCCACATACA CGCTTGAGGC CGTTTATGAA 840

GCCGTCTTCG GTCAGCCGAA GGAGAAGGTT TACGCTGAGG AAATAACACC AGCCTGGGAA 900

ACCGGCGAGA ACCTTGAGAG AGTCGCCCGC TACTCGATGG AAGATGCGAA GGTCACATAC 960

GAGCTTGGGA AGGAGTTCCT TCCGATGGAG GCCCAGCTTT CTCGCTTAAT CGGCCAGTCC 1020

CTCTGGGACG TCTCCCGCTC CAGCACTGGC AACCTCGTTG AGTGGTTCCT CCTCAGGAAG 1080

GCCTATGAGA GGAATGAGCT GGCCCCGAAC AAGCCCGATG AAAAGGAGCT GGCCAGAAGA 1140

CGGCAGAGCT ATGAAGGAGG CTATGTAAAA GAGCCCGAGA GAGGGTTGTG GGAGAACATA 1200

GTGTACCTAG ATTTTAGATC CCTGTACCCC TCAATCATCA TCACCCACAA CGTCTCGCCG 1260

GATACGCTCA ACAGAGAAGG ATGCAAGGAA TATGACGTTG CCCCACAGGT CGGCCACCGC 1320

TTCTGCAAGG ACTTCCCAGG ATTTATCCCG AGCCTGCTTG GAGACCTCCT AGAGGAGAGG 1380

CAGAAGATAA AGAAGAAGAT GAAGGCCACG ATTGACCCGA TCGAGAGGAA GCTCCTCGAT 1440

TACAGGCAGA GGGCCATCAA GATCCTGGCA AACAGCTACT ACGGTTACTA CGGCTATGCA 1500

AGGGCGCGCT GGTACTGCAA GGAGTGTGCA GAGAGCGTAA CGGCCTGGGG AAGGGAGTAC 1560

ATAACGATGA CCATCAAGGA GATAGAGGAA AAGTACGGCT TTAAGGTAAT CTACAGCGAC 1620

ACCGACGGAT TTTTTGCCAC AATACCTGGA GCCGATGCTG AAACCGTCAA AAAGAAGGCT 1680

ATGGAGTTCC TCAACTATAT CAACGCCAAA CTTCCGGGCG CGCTTGAGCT CGAGTACGAG 1740

GGCTTCTACA AACGCGGCTT CTTCGTCACG AAGAAGAAGT ATGCGGTGAT AGACGAGGAA 1800

GGCAAGATAA CAACGCGCGG ACTTGAGATT GTGAGGCGTG ACTGGAGCGA GATAGCGAAA 1860

GAGACGCAGG CGAGGGTTCT TGAAGCTTTG CTAAAGGACG GTGACGTCGA GAAGGCCGTG 1920

AGGATAGTCA AAGAAGTTAC CGAAAAGCTG AGCAAGTACG AGGTTCCGCC GGAGAAGCTG 1980

GTGATCCACG AGCAGATAAC GAGGGATTTA AAGGACTACA AGGCAACCGG TCCCCACGTT 2040

GCCGTTGCCA AGAGGTTGGC CGCGAGAGGA GTCAAAATAC GCCCTGGAAC GGTGATAAGC 2100

TACATCGTGC TCAAGGGCTC TGGGAGGATA GGCGACAGGG CGATACCGTT CGACGAGTTC 2160

GACCCGACGA AGCACAAGTA CGACGCCGAG TACTACATTG AGAACCAGGT TCTCCCAGCC 2220

GTTGAGAGAA TTCTGAGAGC CTTCGGTTAC CGCAAGGAAG ACCTGCGCTA CCAGAAGACG 2280

AGACAGGTTG GTTTGAGTGC TTGGCTGAAG CCGAAGGGAA CTTGA 2325

SEQ. ID NO: 4

LENGTH: 24 base pairs

TYPE: nucleic acid (DNA)

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULAR TYPE: synthetic DNA

SEQUENCE DESCRIPTION:

CTTTTGCTCA GATCTTCTTT CCTG 24

SEQ. ID NO: 5

LENGTH: 24 base pairs

TYPE: nucleic acid (DNA)

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULAR TYPE: synthetic DNA

SEQUENCE DESCRIPTION:

CAGGAAAGAA GATCTGAGCA AAAG 24

SEQ. ID NO: 6

LENGTH: 36 base pairs

TYPE: nucleic acid (DNA)

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULAR TYPE: synthetic DNA

SEQUENCE DESCRIPTION:

CTGAAAATGC TCGCCTTCGC GATTGCAACT CTCTAC 36

SEQ. ID NO: 7

LENGTH: 33 base pairs

TYPE: nucleic acid (DNA)

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULAR TYPE: synthetic DNA

SEQUENCE DESCRIPTION:

CTGAAAATGC TCGCCTTCGC GATTGAAACT CTCT 34

SEQ. ID NO: 8

LENGTH: 30 base pairs

TYPE: nucleic acid (DNA)

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULAR TYPE: synthetic DNA

SEQUENCE DESCRIPTION:

GCCCTCGTGG TAGAGAGTTG CAATGTCGAA 30

SEQ. ID NO: 9

LENGTH: 32 base pairs

TYPE: nucleic acid (DNA)

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULAR TYPE: synthetic DNA

SEQUENCE DESCRIPTION:

CGGACGTACT GATAACGTAC GACGGTGACA AC 32

SEQ. ID NO: 10

LENGTH: 33 base pairs

TYPE: nucleic acid (DNA)

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULAR TYPE: synthetic DNA

SEQUENCE DESCRIPTION:

CGGACGTACT GATAACGTAC GACGGTGACA AC 32

SEQ. ID NO: 11

LENGTH: 35 base pairs

TYPE: nucleic acid (DNA)

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULAR TYPE: synthetic DNA

SEQUENCE DESCRIPTION:

TGGCTAGCCA AGGAACCACC AGTTGATTAG CAGAG 35

SEQ. ID NO: 12

LENGTH: 35 base pairs

TYPE: nucleic acid (DNA)

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULAR TYPE: synthetic DNA

SEQUENCE DESCRIPTION:

ATAAGAGGTC CCAAGACTTA GTACCTGAAG GGTGA 35

SEQ. ID NO: 13

LENGTH: 35 base pairs

TYPE: nucleic acid (DNA)

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULAR TYPE: synthetic DNA

SEQUENCE DESCRIPTION:

AAAAAGTACT CACCAGTCAC AGAAAAGCAT CTTAC 35

SEQ. ID NO: 14

LENGTH: 34 base pairs

TYPE: nucleic acid (DNA)

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULAR TYPE: synthetic DNA

SEQUENCE DESCRIPTION:

AAAAAGTACT CAACCAAGTC ATTCCTGAGA ATAGT 34

SEQ. ID NO: 15

LENGTH: 24 base pairs

TYPE: nucleic acid (DNA)

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULAR TYPE: synthetic DNA

SEQUENCE DESCRIPTION:

CGCCAGGGTT TTCCCAGTCA CGAC 24

SEQ. ID NO: 16

LENGTH: 24 base pairs

TYPE: nucleic acid (DNA)

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULAR TYPE: synthetic DNA

SEQUENCE DESCRIPTION:

CTTTTGCTCA GATCTTCTTT CCTG 24

SEQ. ID NO: 17

LENGTH: 36 base pairs

TYPE: nucleic acid (DNA)

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULAR TYPE: synthetic DNA

SEQUENCE DESCRIPTION:

AGCTGAAAAT GCTAGCCTTC GACAATGAAA CTCTCT 36

SEQ. ID NO: 18

LENGTH: 36 base pairs

TYPE: nucleic acid (DNA)

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULAR TYPE: synthetic DNA

SEQUENCE DESCRIPTION:

AGCTGAAAAT GCTAGCCTTC GACGAAGAAA CTCTCT 36

SEQ. ID NO: 19

LENGTH: 33 base pairs

TYPE: nucleic acid (DNA)

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULAR TYPE: synthetic DNA

SEQUENCE DESCRIPTION:

GAAAATGCTC GCCTTTGATC AAGAAACTCT CTA 33

SEQ. ID NO: 20

LENGTH: 36 base pairs

TYPE: nucleic acid (DNA)

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULAR TYPE: synthetic DNA

SEQUENCE DESCRIPTION:

AGCTGAAAAT GCTAGCCTTC GACGATGAAA CTCTCT 36

SEQ. ID NO: 21

LENGTH: 30 base pairs

TYPE: nucleic acid (DNA)

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULAR TYPE: synthetic DNA

SEQUENCE DESCRIPTION:

CGCCTTCGAC ATTGAAGTAC TCTACCATGA 30

SEQ. ID NO: 22

LENGTH: 36 base pairs

TYPE: nucleic acid (DNA)

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULAR TYPE: synthetic DNA

SEQUENCE DESCRIPTION:

AGCTGAAAAT GCTAGCCTTC GACAGAGAAA CTCTCT 36

SEQ. ID NO: 23

LENGTH: 36 base pairs

TYPE: nucleic acid (DNA)

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULAR TYPE: synthetic DNA

SEQUENCE DESCRIPTION:

AGCTGAAAAT GCTAGCCTTC GACAAAGAAA CTCTCT 36

Claims

1. A modified thermostable DNA polymerase having the following physicochemical properties:

action:	it has a DNA polymerase activity and has 5 % or less of the 3'-5' exonuclease activity of the enzyme before modification;
DNA extension rate:	at least 30 bases/second; and
thermostability:	it is capable of maintaining 60 % or more residual activity at pH 8.8 (determined at 25 °C) after treatment at 95 °C for 6 hours.

2. A modified thermostable DNA polymerase having the following physicochemical properties:

action:	it has 5 % or less of the 3'-5' exonuclease activity of the enzyme before modification;
DNA extension rate:	at least 30 bases/second;
thermostability:	it is capable of maintaining residual activity at pH 8.8 (determined at 25 °C) after treatment at 95 °C for 6 hours;
optimum temperature:	about 75 °C;
molecular weight:	88 to 90 kDa; and
amino acid sequence:	an amino acid sequence as shown in SEQ ID NO: 2 in which at least one of amino acids at the 141-, 143-, 210- and 311-positions has been replaced by another amino acid.

3. A modified thermostable DNA polymerase having the following physicochemical properties:

action:	it has a DNA polymerase activity and is free of a 3'-5' exonuclease activity;
DNA extension rate:	at least 30 bases/second;
thermostability:	it is capable of maintaining 60 % or more residual activity at pH 8.8 (determined at 25 °C) after treatment at 95 °C for 6 hours;
optimum temperature:	about 75 °C;
molecular weight:	88 to 90 kDa; and
amino acid sequence:	an amino acid sequence as shown in SEQ ID NO: 2 in which at least one of amino acids at the 141-, 143-, 210- and 311-positions has been replaced by another amino acid.

4. The thermostable DNA polymerase according to any one of claims 1 to 3, wherein the DNA extension rate is not less than 60 bases/second.

5. The thermostable DNA polymerase according to claim 1 or 2, wherein the 3'-5'exonuclease activity is reduced to about 1 % or less.

6. The thermostable DNA polymerase according to any one of claims 1 to 3, wherein in SEQ ID NO: 2 aspartic acid at the 141-position has been replaced by another amino acid.

7. The thermostable DNA polymerase according to claim 6, wherein in SEQ ID NO: 2 aspartic acid at the 141-position has been replaced by alanine.

8. The thermostable DNA polymerase according to any one of claims 1 to 3, wherein in SEQ ID NO: 2 glutamic acid at the 143-position has been replaced by another amino acid.

9. The thermostable DNA polymerase according to claim 8, wherein in SEQ ID NO: 2 glutamic acid at the 143-position has been replaced by alanine.

10. The thermostable DNA polymerase according to any one of claims 1 to 3, wherein in SEQ ID NO: 2 aspartic acid at the 141-position and glutamic acid at the 143-position have been replaced by other amino acids.

11. The thermostable DNA polymerase according to claim 10, wherein in SEQ ID NO: 2 aspartic acid at the 141-position and glutamic acid at the 143-positoin have been replaced by alanine.

12. The thermostable DNA polymerase according to any one of claims 1 to 3, wherein in SEQ ID NO: 2 asparagine at the 210-position has been replaced by another amino acid.

13. The thermostable DNA polymerase according to claim 12, wherein in SEQ ID NO: 2 asparagine at the 210-position has been replaced by aspartic acid.

14. The thermostable DNA polymerase according to any one of claims 1 to 3, wherein in SEQ ID NO: 2 tyrosine at the 311-position has been replaced by another amino acid.

15. The thermostable DNA polymerase according to claim 14, wherein in SEQ ID NO: 2 tyrosine at the 311-position has been replaced by phenylalanine.

16. A method for amplifying nucleic acid, which comprises reacting DNA as a template, primers, dNTP and the thermostable DNA polymerase of claims 1 to 3, thus extending the primers to synthesize a DNA primer extension product.

17. The method for amplifying nucleic acid according to claim 16, wherein the primers are 2 kinds of oligonucleotide, one of which is complementary to a DNA extension product of another primer.

18. The method for amplifying nucleic acid according to claim 16, wherein heating and cooling are repeatedly carried out.

19. A reagent for amplifying nucleic acid, which comprises 2 kinds of primer, one of which is complementary to a DNA extension product of another primer, dNTP, the thermostable DNA polymerase of claims 1 to 3, and a buffer solution.

20. A reagent for amplifying nucleic acid, which comprises 2 kinds of primer, one of which is complementary to a DNA extension product of another primer, dNTP, the thermostable DNA polymerase of claims 1 to 3, magnesium ions, ammonium ions and/or potassium ions, BSA, a nonionic surface active agent, and a buffer solution.

21. A DNA polymerase composition for amplifying nucleic acid, which comprises a modified thermostable DNA polymerase having 0 to 5 % of the 3'-5' exonuclease activity of the enzyme before modification (first polymerase) and a thermostable DNA polymerase having the 3'-5' exonuclease activity or a modified thermostable DNA polymerase having 100 to 6 % of the 3'-5' exonuclease activity of a thermostable DNA polymerase before modification (second polymerase), said first and second DNA polymerases having a DNA extension rate of at least 30 bases/second and capable of maintaining 60 % or more residual activity at pH 8.8 (determined at 25 °C) after treatment at 95 °C for 6 hours.

22. The DNA polymerase composition for amplifying nucleic acid according to claim 21, wherein the activity of the second DNA polymerase is lower than the activity of the first DNA polymerase.

23. The DNA polymerase composition for amplifying nucleic acid according to claim 21, wherein the second DNA polymerase is present in 0.02 to 0.1 unit every 2.5 units of the first DNA polymerase.

24. The DNA polymerase composition according to claim 21, wherein the 3'-5' exonuclease activity of the first DNA polymerase is reduced to about 1 % or less of the 3'-5' exonuclease activity of the thermostable DNA polymerase before modification.

25. The DNA polymerase composition according to claim 21, wherein the first DNA polymerase is a modified ther-

mostable DNA polymerase having the following physicochemical properties:

action:	it has a DNA polymerase activity and has 0 to 5 % of the 3'-5' exonuclease activity of the enzyme before modification;
DNA extension rate:	at least 30 bases/second; and
thermostability:	it is capable of maintaining 60 % or more residual activity at pH 8.8 (determined at 25 °C) after treatment at 95 °C for 6 hours.

26. The DNA polymerase composition according to claim 21, wherein the first DNA polymerase is a modified thermostable DNA polymerase having the following physicochemical properties:

action:	it has a DNA polymerase activity and has 0 to 5 % of the 3'-5' exonuclease activity of the enzyme before modification;
DNA extension rate:	at least 30 bases/second;
thermostability:	it is capable of maintaining 60 % or more residual activity at pH 8.8 (determined at 25 °C) after treatment at 95 °C for 6 hours;
optimum temperature:	about 75 °C;
molecular weight:	88 to 90 kDa; and
amino acid sequence:	
	an amino acid sequence as shown in SEQ ID NO: 2 in which at least one of amino acids at the 141-, 142-, 143-, 210- and 311-positions has been replaced by another amino acid.

27. The DNA polymerase composition according to claim 21, wherein the first DNA polymerase is a modified thermostable DNA polymerase having the following physicochemical properties:

action:	it has a DNA polymerase activity and has 0 to 5 % of the 3'-5' exonuclease activity of the enzyme before modification;
DNA extension rate:	at least 30 bases/second;
thermostability:	it is capable of maintaining 60 % or more residual activity at pH 8.8 (determined at 25 °C) after treatment at 95 °C for 6 hours;
optimum temperature:	about 75 °C;
molecular weight:	88 to 90 kDa; and
amino acid sequence:	
	an amino acid sequence as shown in SEQ ID NO: 2 in which aspartic acid at the 141-position has been replaced by alanine, isoleucine at the 142-position by arginine, glutamic acid at the 143-position by alanine, aspartic acid at the 141-position and glutamic acid at the 143-position respectively by alanine, asparagine at the 210-position by aspartic acid, and tyrosine at the 311-position by phenylalanine.

28. The DNA polymerase composition according to claim 21, wherein the first DNA polymerase is a thermostable DNA polymerase as shown in SEQ ID NO: 2 in which aspartic acid at the 141-position has been replaced by alanine.

29. The DNA polymerase composition according to claim 21, wherein the first DNA polymerase is a thermostable DNA polymerase as shown in SEQ ID NO: 2 in which isoleucine at the 142-position has been replaced by arginine.

30. The DNA polymerase composition according to claim 21, wherein the first DNA polymerase is a thermostable DNA polymerase as shown in SEQ ID NO: 2 in which glutamic acid at the 143-position has been replaced by alanine.

31. The DNA polymerase composition according to claim 21, wherein the first DNA polymerase is a thermostable DNA polymerase as shown in SEQ ID NO: 2 in which aspartic acid at the 141-position and glutamic acid at the 143-position have been replaced respectively by alanine.

32. The DNA polymerase composition according to claim 21, wherein the first DNA polymerase is a thermostable DNA polymerase as shown in SEQ ID NO: 2 in which asparagine at the 210-position has been replaced by aspartic acid.

33. The DNA polymerase composition according to claim 21, wherein the first DNA polymerase is a thermostable DNA polymerase as shown in SEQ ID NO: 2 in which tyrosine at the 311-position has been replaced by phenylalanine.

34. The DNA polymerase composition according to claim 21, wherein the second polymerase is a thermostable DNA polymerase having the following physicochemical properties:

action:	it has a DNA polymerase activity and has a 3'-5' exonuclease activity;
DNA extension rate:	at least 30 bases/second;
thermostability:	it is capable of maintaining 60 % or more residual activity at pH 8.8 (determined at 25 °C) after treatment at 95 °C for 6 hours;
optimum temperature:	about 75 °C;
molecular weight:	88 to 90 kDa; and
amino acid sequence:	
	the amino acid sequence of SEQ ID NO: 2.

35. The DNA polymerase composition according to claim 21, wherein the second polymerase is a modified thermostable DNA polymerase having the following physicochemical properties:

action:	it has a DNA polymerase activity and has 100 to 6 % of the 3'-5' exonuclease activity of the enzyme before modification;
DNA extension rate:	at least 30 bases/second;
thermostability:	it is capable of maintaining 60 % or more residual activity at pH 8.8 (determined at 25 °C) after treatment at 95 °C for 6 hours; and
amino acid sequence:	
	an amino acid sequence as shown in SEQ ID NO: 2 in which at least one of amino acids X_1, X_2 and X_3 in an $X_1DX_2EX_3$ motif present in EXO 1 has been replaced by another amino acid.

36. The DNA polymerase composition according to claim 21, wherein the second DNA polymerase is a modified thermostable DNA polymerase having the following physicochemical properties:

action:	it has a DNA polymerase activity and has 100 to 6 % of the 3'-5' exonuclease activity of the enzyme before modification;
DNA extension rate:	at least 30 bases/second;
thermostability:	it is capable of maintaining 60 % or more residual activity at pH 8.8 (determined at 25 °C) after treatment at 95 °C for 6 hours; and
amino acid sequence:	
	an amino acid sequence as shown in SEQ ID NO: 2 in which amino acids at the 140-, 142- and 144-positions have been replaced by other amino acids.

37. The DNA polymerase composition according to claim 21, wherein the second DNA polymerase is a modified thermostable DNA polymerase having the following physicochemical properties:

action:	it has a DNA polymerase activity and has 100 to 6 % of the 3'-5' exonuclease activity of the enzyme before modification;
DNA extension rate:	at least 30 bases/second;
thermostability:	it is capable of maintaining 60 % or more residual activity at pH 8.8 (determined at 25 °C) after treatment at 95 °C for 6 hours;
optimum temperature:	about 75 °C;
molecular weight:	88 to 90 KDa; and
amino acid sequence:	
	an amino acid sequence as shown in SEQ ID NO: 2 in which is oleucine at the 142-position has been replaced by aspartic acid, glutamic acid, asparagine, glutamine or lysin, or threonine at the 144-position by valine.

38. The DNA polymerase composition according toe claim 21, wherein the second DNA polymerase is a thermostable DNA polymerase as shown in SEQ ID NO: 2 in which isoleucine at the 142-position has been replaced by aspartic acid.

39. The DNA polymerase composition according to claim 21, wherein the second DNA polymerase is a thermostable DNA polymerase as shown in SEQ ID NO: 2 in which isoleucine at the 142-position has been replaced by glutamic

acid.

40. The DNA polymerase composition according to claim 21, wherein the second DNA polymerase is a thermostable DNA polymerase as shown in SEQ ID NO: 2 in which isoleucine at the 142-position has been replaced by asparagine.

41. The DNA polymerase composition according to claim 21, wherein the second DNA polymerase is a thermostable DNA polymerase as shown in SEQ ID NO: 2 in which isoleucine at the 142-position has been replaced by glutamine.

42. The DNA polymerase composition according to claim 21, wherein the second DNA polymerase is a thermostable DNA polymerase as shown in SEQ ID NO: 2 in which isoleucine at the 142-position has been replaced by lysin.

43. The DNA polymerase composition according to claim 21, wherein the second DNA polymerase is a thermostable DNA polymerase as shown in SEQ ID NO: 2 in which isoleucine at the 142-position has been replaced by arginine.

44. The DNA polymerase composition according to claim 21, wherein the second DNA polymerase is a thermostable DNA polymerase as shown in SEQ ID NO: 2 in which threonine at the 144-position has been replaced by valine.

45. A DNA polymerase composition for amplifying nucleic acid, which comprises the following first and second DNA polymerases:

the first polymerase:

action:	it has a DNA polymerase activity and has 0 to 5 % of the 3'-5' exonuclease activity of the enzyme before modification;
DNA extension rate:	at least 30 bases/second;
thermostability:	it is capable of maintaining 60 % or more residual activity at pH 8.8 (determined at 25 °C) after treatment at 95 °C for 6 hours;
optimum temperature:	about 75 °C;
molecular weight:	88 to 90 kDa; and
amino acid sequence:	an amino acid sequence as shown in SEQ ID NO: 2 in which aspartic acid at the 141-position has been replaced by alanine, isoleucine at the 142-position by arginine, glutamic acid at the 143-position by alanine, aspartic acid at the 141-position and glutamic acid at the 143-position respectively by alanine, asparagine at the 210-position by aspartic acid, or tyrosine at the 311-position by phenylalanine,

the second polymerase:

action:	it has a DNA polymerase activity and has a 3'-5' exonuclease activity;
DNA extension rate:	at least 120 bases/second;
thermostability:	it is capable of maintaining 60 % or more residual activity at pH 8.8 (determined at 25 °C) after treatment at 95 °C for 6 hours;
optimum temperature:	about 75 °C;
molecular weight:	88 to 90 kDa; and
amino acid sequence:	the amino acid sequence of SEQ ID NO: 2, or
action:	it has a DNA polymerase activity and has 100 to 30 % of the 3'-5' exonuclease activity of the enzyme before modification;
DNA extension rate:	at least 120 bases/second;
thermostability:	it is capable of maintaining 60 % or more residual activity at pH 8.8 (determined at 25 °C) after treatment at 95 °C for 6 hours;
optimum temperature:	about 75 °C;
molecular weight:	88 to 90 kDa; and
amino acid sequence:	an amino acid sequence as shown in SEQ ID NO: 2 in which is oleucine at the 142-position has been replaced by aspartic acid, glutamic acid,asparagine, glutamine or

lysin, or threonine at the 144-position by valine.

46. A DNA polymerase composition for amplifying nucleic acid, which comprises the following first and second DNA polymerases:

the first polymerase:

action:	it has a DNA polymerase activity and has 0 to 5 % of the 3'-5' exonuclease activity of the enzyme before modification;
DNA extension rate:	at least 30 bases/second;
thermostability:	it is capable of maintaining 60 % or more residual activity at pH 8.8 (determined at 25 °C) after treatment at 95 °C for 6 hours;
optimum temperature:	about 75 °C;
molecular weight:	88 to 90 kDa; and
amino acid sequence:	an amino acid sequence as shown in SEQ ID NO: 2 in which asparagine at the 210-position by aspartic acid,

the second polymerase:

action:	it has a DNA polymerase activity and has a 3'-5' exonuclease activity;
DNA extension rate:	at least 120 bases/second;
thermostability:	it is capable of maintaining 60 % or more residual activity at pH 8.8 (determined at 25 °C) after treatment at 95 °C for 6 hours;
optimum temperature:	about 75 °C;
molecular weight:	88 to 90 kDa; and
amino acid sequence:	the amino acid sequence of SEQ ID NO: 2.

47. A method for amplifying nucleic acid, which comprises reacting DNA as a template, primers, dNTP and the DNA polymerase composition of any one of claims 21 to 46, thus extending the primers to synthesize a DNA primer extension product.

48. The method for amplifying nucleic acid according to claim 46 or 47, wherein the primers are 2 kinds of oligonucleotide, one of which is complementary to a DNA extension product of another primer.

49. The method for amplifying nucleic acid according to claim 46 or 47, wherein heating and cooling are repeatedly carried out.

50. A reagent for amplifying nucleic acid, which comprises the DNA polymerase composition of any one of claims 21 to 46, divalent ions, monovalent ions, primers, dNTP, and a buffer solution.

51. A reagent for amplifying nucleic acid, which comprises the DNA polymerase composition of any one of claims 21 to 46, magnesium ions, ammonium ions and/or potassium ions, 2 kinds of primer, one of which is complementary to a DNA extension product of another primer, dNTP, BSA, a nonionic surface active agent, and a buffer solution.

FIG. 1

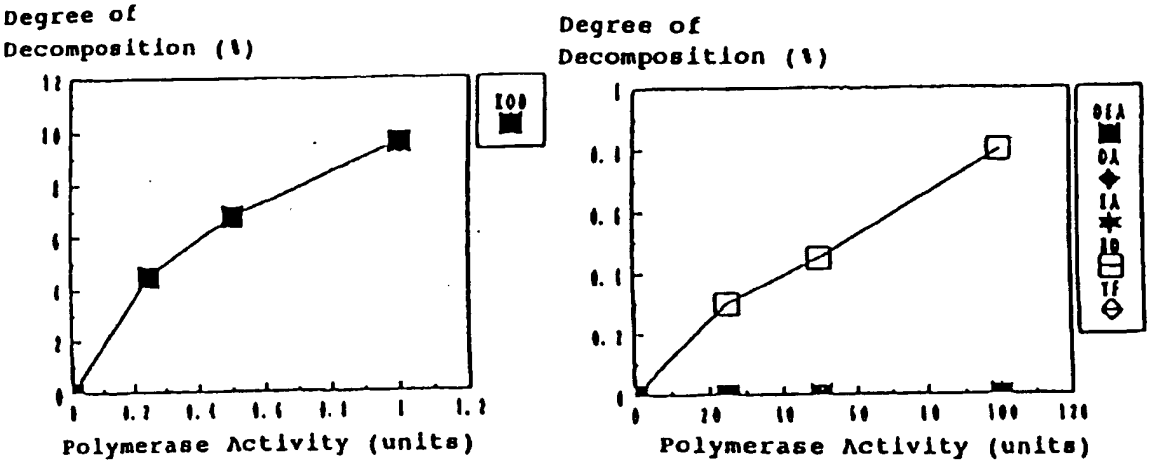

Degree of Decomposition (%)
Polymerase Activity (units)
Degree of Decomposition (%)
Polymerase Activity (units)

FIG. 2

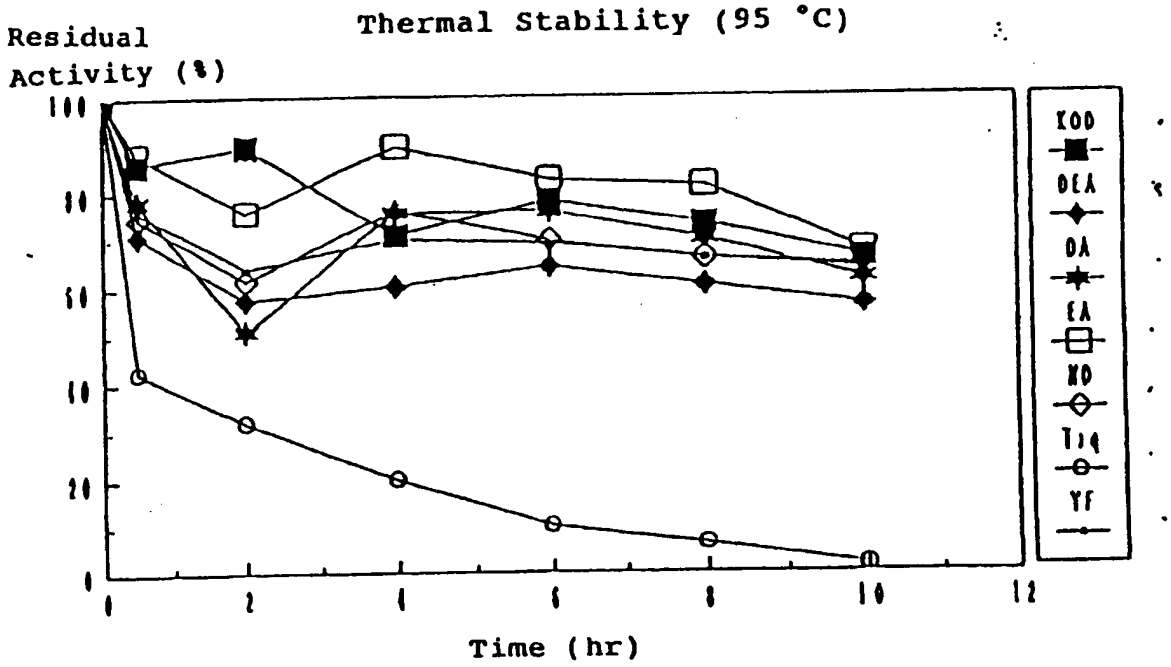
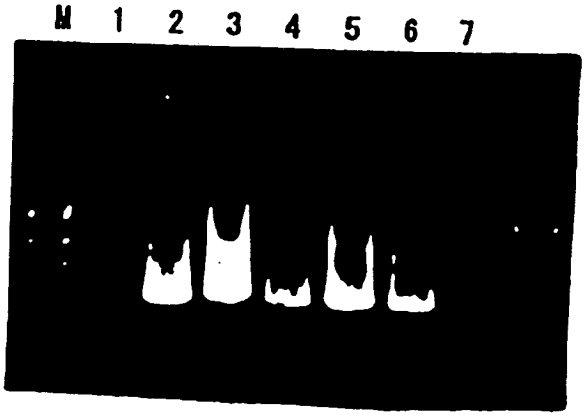
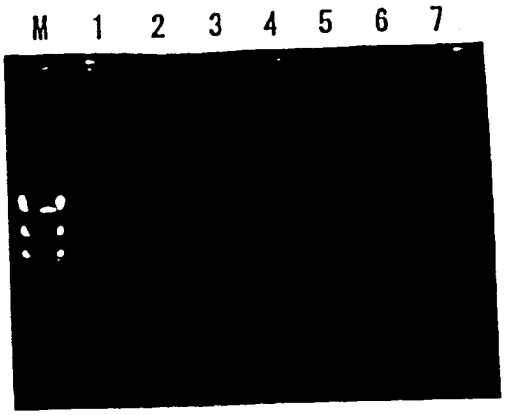

Thermal Stability (95 °C)
Residual Activity (%)
Time (hr)

FIG. 3



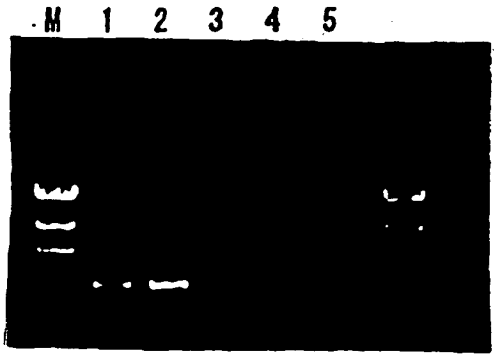
Lane1:KOD
2:YF
3:ND
4:EA
5:DEA
6:DA
7:Taq
M:λ/HindIII Marker

FIG. 4



Lane 1 : YF
2 : ND
3 : EA
4 : DEA
5 : EA
6 : Taq
7 : KOD
M : λ / HindIII Marker

FIG. 5



Lane 1: ND
2: ND+KOD
3: Advantage Tthmix (Clontech)
4: Ex Taq (Takara)
5: Taq (Toyobo)
M: λ/HindIII Marker

FIG. 6

	EXO I	EXO II	EXO III
KOD	MLAFDIETLY	LITYNGDNFDFAYLKKR	YARYSWEDAKY
Pfu	ILAFDIETLY	IYTYNGDSFDFPYLAKR	YAKYSWEDAKA
Vent	LLAFDIETFY	IITYNGDNFDLPYLIKR	YAKYSWEDAKA
Deep Vent	LLAFDIETLY	IITYNGDSFDLPYLPKR	YAKYSWEDAKY

FIG. 7

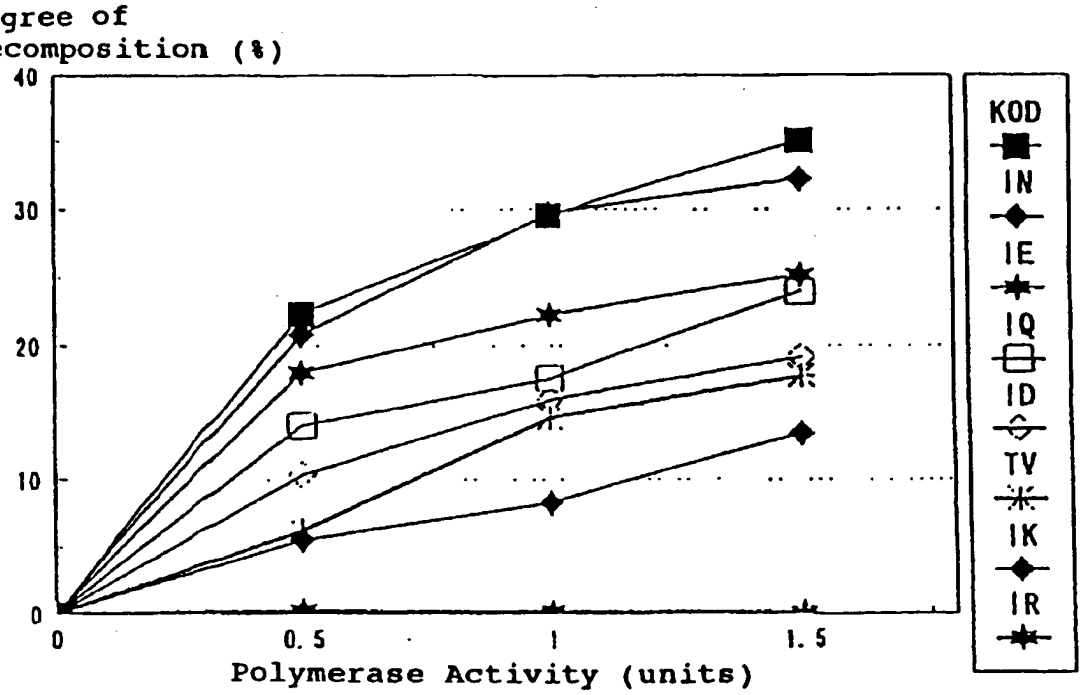
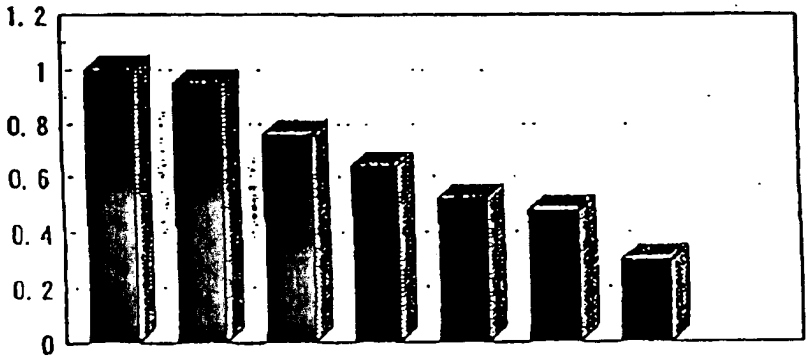


FIG. 8



	wKOD	IN	IE	IQ	ID	TV	IK	IR
Relative Activity	1.00	0.95	0.76	0.64	0.52	0.48	0.30	0.00

FIG. 1

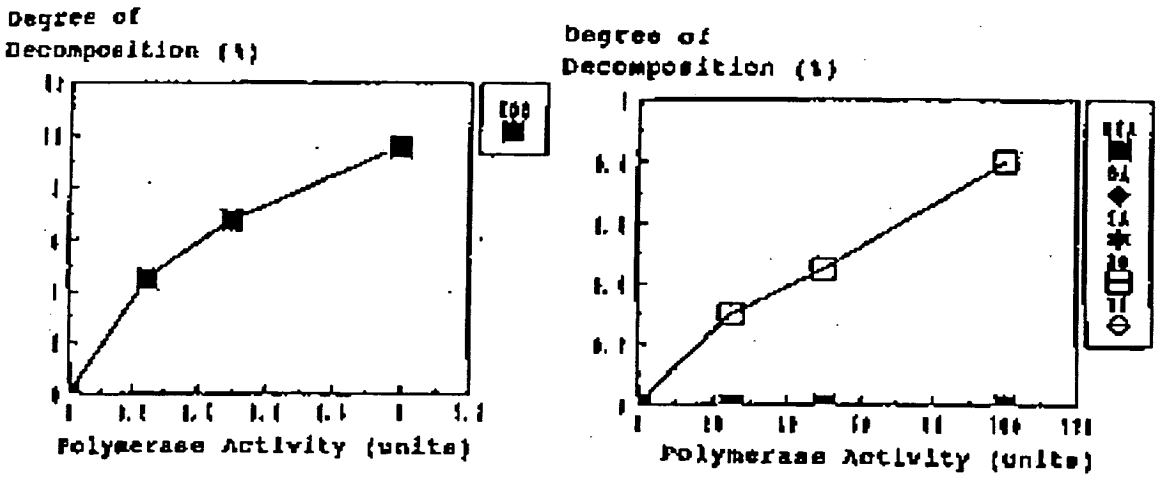

Degree of Decomposition (%)
Polymerase Activity (units)
Degree of Decomposition (%)
Polymerase Activity (units)

FIG. 2

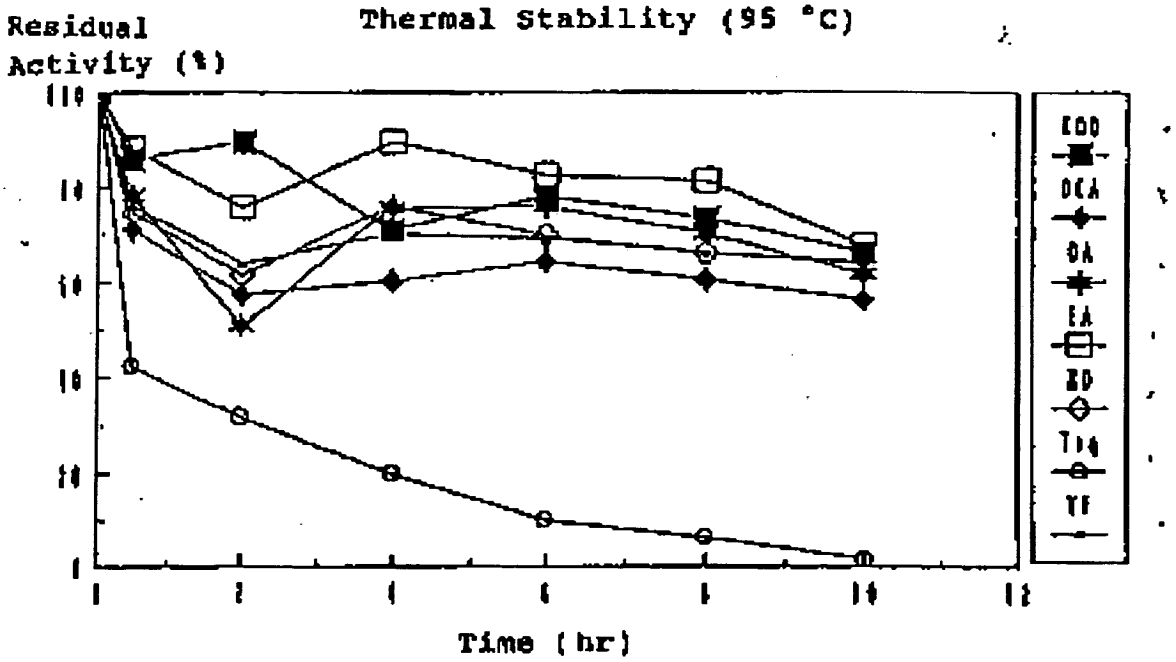
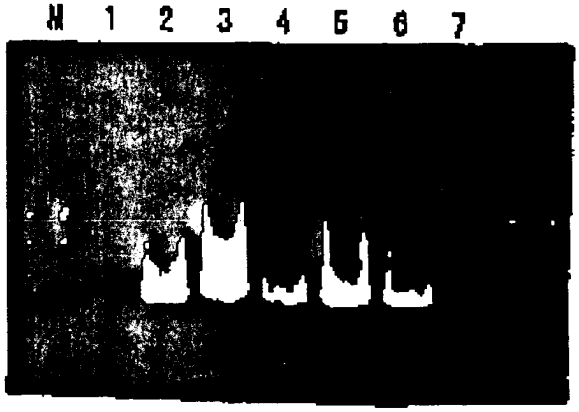
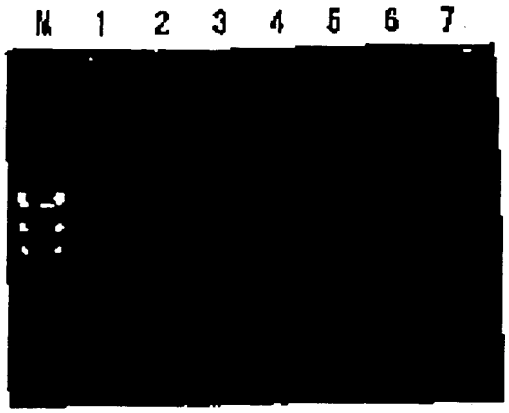

Thermal Stability (95 °C)
Residual Activity (%)
Time (hr)

FIG. 3



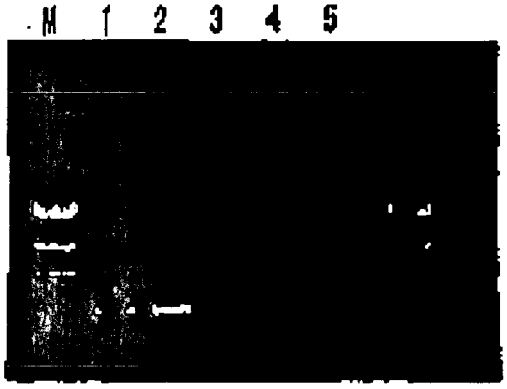
Lane 1: XOD
2: YF
3: MD
4: EA
5: DEA
6: DA
7: Taq
M: λ/HindIII Marker

FIG. 4



Lane 1: YF
2: KD
3: EA
4: DEA
5: EA
6: Taq
7: KOD
M: λ/HindIII Marker

FIG. 5



Lane1:ND
2:ND+KOD
3:Advantage Tthmix (Clontech)
4:Ex Taq (Takara)
5:Taq (Toyobo)
M:λ/HindIII Marker

FIG. 6

	EXO I	EXO II	EXO III
KOD	MLAFDIETLY	LITYMGDNFDFAYLKKR	YARYSWEDAKV
Pfu	ILAFDIETLY	IVTYMGDSFDFPYLAKK	YAKYSWEDAKA
Vent	LLAFDIETPY	IITYMGDNFDLPYLIKR	YAKYSWEDAKA
Deep Vent	LLAFDIETLY	IITYMGDSFDLPYLPKR	YAKYSWEDAKV

FIG. 7

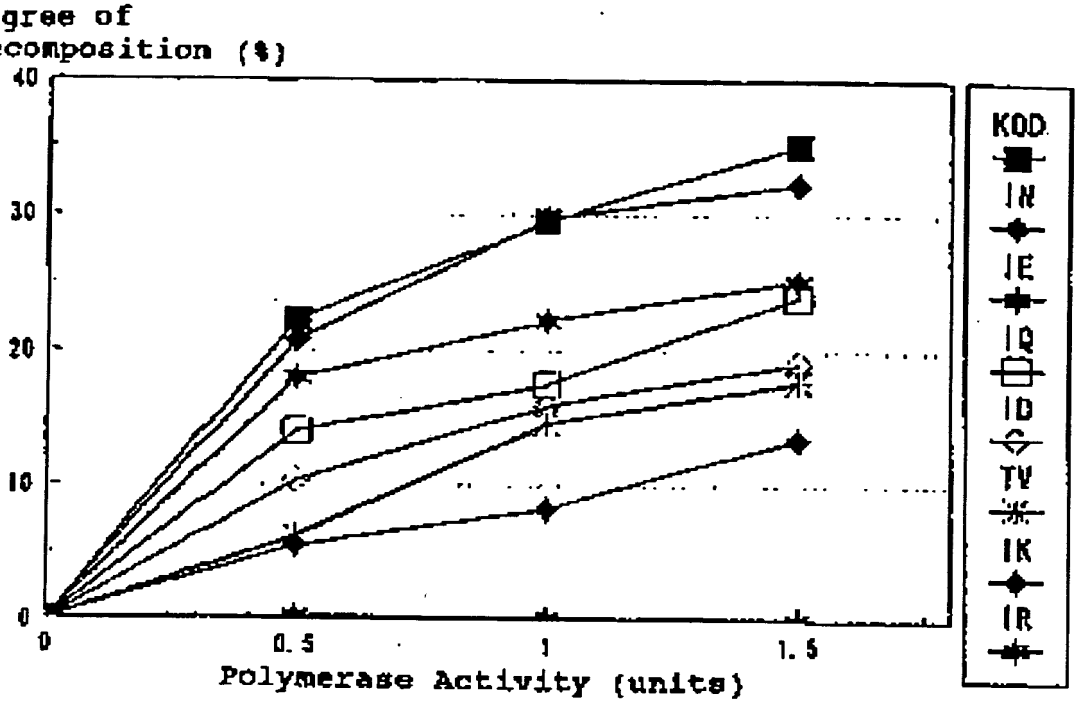
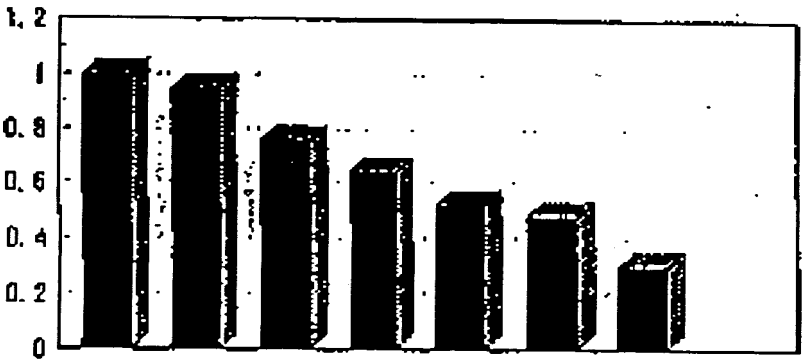


FIG. 8



	wKOD	IN	IE	IQ	ID	TV	IK	IR
Relative Activity	1.00	0.95	0.76	0.64	0.52	0.48	0.30	0.00

(19) Europäisch s Pat ntamt
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(54) Modified thermostable DNA polymerase, and DNA polymerase composition for nucleic acid amplification

(57) A modified thermostable DNA polymerase having 5 % or less of the 3'-5' exonuclease activity of the enzyme before modification and a DNA polymerase composition for amplifying nucleic acid, which comprises the modified thermostable DNA polymerase having 0 to 5 % of the 3'-5' exonuclease activity of the enzyme before modification and an unmodified thermostable DNA polymerase having 3'-5' exonuclease activity or a modified thermostable DNA polymerase having 100 to 6 % of the 3'-5' exonuclease activity of a thermostable DNA polymerase before modification; a method for amplifying nucleic acid by use of said modified thermostable polymerase or said DNA polymerase composition; and a reagent therefor.

EP 0 822 256 A3

European Patent Office

EUROPEAN SEARCH REPORT

Application Number
EP 97 11 2760

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
A	MORIKAWA M ET AL: "PURIFICATION AND CHARACTERIZATION OF A THERMOSTABLE THIOL PROTEASE FROM A NEWLY ISOLATED HYPERTHERMOPHILIC PYROCOCCUS SP" APPLIED AND ENVIRONMENTAL MICROBIOLOGY,US,WASHINGTON,DC, vol. 60, no. 12, 1 December 1994 (1994-12-01), pages 4559-4566, XP000673005 ISSN: 0099-2240 * the whole document *		C12N15/54 C12N9/12 C12Q1/68
A	KITABAYASHI M ET AL: "KOD DNA POLYMERASE APPLICATION FOR FAST AND ACCURATE PCR" FASEB JOURNAL,US,FED. OF AMERICAN SOC. FOR EXPERIMENTAL BIOLOGY, BETHESDA, MD, vol. 10, no. 6, 30 April 1996 (1996-04-30), page A1245 XP002017308 ISSN: 0892-6638 Abstract no. 1423; * abstract *		
P,A	EP 0 745 675 A (TOYO BOSEKI) 4 December 1996 (1996-12-04) * the whole document *		
A	WO 94 26766 A (BARNES WAYNE M) 24 November 1994 (1994-11-24) * the whole document *		
A	WO 95 16028 A (STRATAGENE INC) 15 June 1995 (1995-06-15) * the whole document *		
A	EP 0 669 401 A (HOFFMANN LA ROCHE) 30 August 1995 (1995-08-30) * the whole document *		

-/--

TECHNICAL FIELDS SEARCHED (Int.Cl.6)
C12N
C12Q

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	23 January 2001	Hornig, H

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EUROPEAN SEARCH REPORT

Application Number
EP 97 11 2760

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
A	US 5 489 523 A (MATHUR ERIC J) 6 February 1996 (1996-02-06) * the whole document *		

TECHNICAL FIELDS SEARCHED (Int.Cl.6)

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23-01-2001

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0745675 A	04-12-1996	JP 8322597 A	10-12-1996
		US 6143536 A	07-11-2000
		US 6054301 A	25-04-2000
WO 9426766 A	24-11-1994	US 5436149 A	25-07-1995
		AT 181573 T	15-07-1999
		AU 671204 B	15-08-1996
		AU 6246494 A	12-12-1994
		CA 2156176 A	24-11-1994
		DE 69419248 D	29-07-1999
		DE 69419248 T	27-01-2000
		DK 693078 T	24-01-2000
		EP 0693078 A	24-01-1996
		ES 2136730 T	01-12-1999
		JP 11239492 A	07-09-1999
		JP 2885324 B	19-04-1999
		JP 11501801 T	16-02-1999
		NZ 262663 A	22-09-1997
WO 9516028 A	15-06-1995	US 5556772 A	17-09-1996
EP 0669401 A	30-08-1995	US 5512462 A	30-04-1996
		CA 2143229 A	26-08-1995
		JP 8038198 A	13-02-1996
US 5489523 A	06-02-1996	US 5948663 A	07-09-1999
		US 5545552 A	13-08-1996
		WO 9209689 A	11-06-1992
		US 5866395 A	02-02-1999

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